

Luisa L. Rocha
Alberto Lazarowski
Esper A. Cavalheiro *Editors*

Pharmacoresistance in Epilepsy

From Genes and Molecules to Promising
Therapies

Second Edition

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Preface

Epilepsy is a neurological problem that has afflicted humanity forever. At present, it is known that about 50 million persons have epilepsy. A relevant situation is that timely diagnosis and treatment of this disorder would allow 70% of patients to live without epileptic activity. Antiseizure medications have been obtained since the end of the nineteenth century. They have been identified for generations depending on their mechanism. Currently around 30 antiseizure medications are in use, most of which have been approved within the last 30 years. Despite the use of old and new antiseizure medications, 30% of patients with epilepsy are drug-resistant, a condition associated with comorbid disorders and stigmatization. The treatment of drug-resistant epilepsy is a challenge and requires a structured multidisciplinary approach, especially in regions with low economical resources where 80% of patients with epilepsy are living.

The present book includes the analysis and description of a broad spectrum of molecular mechanisms, functional and structural alterations that characterize drug-resistant epilepsies. The chapters are focused to support that drug resistance in epilepsy is associated with the presence of refractory syndromes from the start. They also include information that the drug-resistant phenotype can be acquired as a consequence of the seizures themselves. Information confirms that genetic differences may impact the pharmacokinetics of antiseizure medications. Also, several haplotypes in the polymorphisms of enzymes and transporters can explain the different inter-individual and/or inter-ethnic responses to the antiseizure medications. This book also contains information about pharmacodynamics aspects, as changes in the location and expression of receptors in the brain, that can explain the drug-resistant condition in epilepsy. Furthermore, it is indicated that different situations, such as hypoxia, neuroinflammation, and oxidative stress, can facilitate the drug-resistant phenotype. In addition, experimental evidence is included to support that drug-resistant epilepsy can compromise peripheral organs such as the heart, with a high risk of sudden unexpected death.

Chapters also contain information about different non-pharmacological therapeutic strategies to control drug-resistant epilepsy, including neurostimulation, new surgical techniques, ketogenic diet, etc. It is also indicated the relevance of novel

omics technologies to the identification of new therapeutic targets that will help to control and prevent drug-resistant epilepsy and its long-term consequences. This book includes information about different experimental models useful to investigate different aspects associated with drug-resistant seizures and epilepsy.

We really hope that this book will result in new perspectives about the drug-resistant condition in epilepsy that improve its knowledge. This situation will improve the quality of life of the persons suffering epilepsy and prevent the drug-resistant condition, especially in countries with low economical resources.

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Chapter 1

Why Study Drug-Resistant Epilepsy?



Luisa L. Rocha, Esper A. Cavalheiro, and Alberto Lazarowski

Abstract Drug-resistant epilepsy (DRE) represents an important challenge because currently available pharmacological therapies fail to control this condition. This situation can be explained because the mechanisms underlying the drug-resistant condition in epilepsy are not addressed correctly.

The high prevalence of patients with DRE (30%) can be associated with the presence of refractory syndromes from the start. Also, drug resistance in epilepsy can be acquired as consequence of the seizure activity at high frequency and severity. Genetic factors may impact the pharmacokinetics and the inter-individual and/or interethnic responses to the antiseizure medications (ASMs). Pharmacodynamic aspects associated to ASMs targets can also explain the pharmacoresistant condition in epilepsy. The investigation of these conditions and their consequences (hypoxia, inflammation, oxidative stress, etc.) in the brain and peripheral organs is essential to design more effective therapeutic strategies to control DRE. Indeed, the use of omics technologies can contribute to the identification of novel targets to control DRE. Also, it is essential in the use of preclinical models that reproduce the different types of pharmacoresistant epilepsies in patients.

The importance of studying DRE lies on the understanding the heterogeneous mechanisms involved and consider new and more effective therapeutic strategies to control and prevent this condition.

Keywords Drug-resistant epilepsy · Antiseizure medications · Pharmacodynamics · Pharmacokinetics · Comorbidities

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Drug-resistant epilepsy (DRE), characterized by failure of adequate trials of two tolerated and appropriately chosen and used antiseizure medications (whether as monotherapies or in combination) to achieve sustained seizure freedom (Kwan et al. 2010), has been a challenge for everyone who faces this diagnosis, whether as professionals who deal with epileptology or as patients and families who must fight with it in their daily lives. Under different labels such as pharmaco-resistant epilepsy, drug refractory epilepsy or others, and despite all efforts to find more effective drugs and alternative treatments, DRE continues to be present in neurology clinics with the same prevalence (15 and 30% according to different studies) as it did decades ago. Some studies indicate that approximately 20% of people with generalized epilepsies and 30% of those with focal epilepsies may eventually develop DRE. In some people, DRE can be identified in the first years of the epileptic condition, in others it can appear after years of controlled seizures and in still others, periods of improvement alternate with periods in which antiseizure medications fail again (see Chap. 2).

These initial observations alone justify the urgent need to comprehend the underlying mechanisms of DRE. Consequently, the specialized literature has grown exponentially as well as new methodologies that have allowed exploring new biological paths, including omics sciences, imaging studies, etc. The search also for new therapeutic approaches, as neurostimulation, new surgical techniques, ketogenic diet, etc., has brought new hope to patients in the perspective of finding improvements in their quality of life. A nonexhaustive search in PubMed on the term DRE shows that the number of publications jumped from 150/year between 1995 and 2000, to almost 2000/year between 2017 and 2022. The number of symposiums, meetings, or regional discussions and the resulting publication of books oriented to the understanding of the DRE have also increased considerably in the last decade. Studies have focused on various aspects of the possible pathophysiological mechanisms of DRE, and on the search of biomarkers that may indicate which people are subject to developing DRE. The advancement of this knowledge brings the expectation that they will act as solid foundations for new therapeutic approaches.

The most recent literature also indicates other hypotheses that can help solve the puzzle that DRE represents. One of them suggests that epilepsy-associated structural alterations could contribute to the formation of abnormal neural networks altering the targets of antiseizure medications and could lead to a reduction in treatment efficacy. An interesting perspective about how we must address the study of DRE is to accept the complexity of this disorder and establish new hypotheses and concepts of brain organization (see Chap. 4). Indeed, the complexity of DRE involves changes in peripheral organs such as heart, a condition that can explain the sudden unexpected death in epilepsy (SUDEP) (see Chap. 6).

Some authors have suggested that clinical characteristics observed in the initial presentation of the epileptic condition may indicate the later development of DRE. Relevant indicators are abnormal EEG findings (see Chap. 5), younger age of onset, neurological deficits, comorbidities at the time of diagnosis (see Chap. 18), symptomatic etiology, high frequency of severe seizures, and nonresponse to the first anticonvulsant. While this information may be useful in the current search for

precision and patient-oriented interventions, it does not provide adequate clues to unravel its underlying mechanisms.

The neurobiological basis of DRE has proven to be very complex, and it is quite likely that the interaction between different mechanisms plays an important role. Some chapters of the present book focus to present some of these mechanisms as they have been the subject of in-depth analysis in several recent studies. Novel mechanisms that result in changes of the targets to antiseizure medications are considered to explain the DRE (see Chap. 7). For example, studies support significant changes in the location and composition of GABA_A receptors in the brain of subjects with DRE. Indeed, experimental evidence support that the activation of GABA_A receptors under specific conditions may induce excitatory effects with changes in the response to some antiseizure medications (see Chap. 16).

Hypoxia-ischemic events associated with severe seizures may affect different types of cells, organs, and systems. The seizure-induced hypoxia-ischemic events can lead to the already well-characterized “epileptic heart” that facilitates SUDEP (see Chap. 11). Furthermore, recurrent seizure activity leads to a process of chronic neuroinflammation and excitotoxicity that facilitate the drug-resistant condition (see Chaps. 8, 12, and 14). In addition, the seizure activity induces cerebrovascular remodeling and changes in the expression of vascular endothelial growth factors that may contribute to DRE phenotype (see Chap. 13).

One mechanism of resistance to antiseizure medications is associated with the overexpression of efflux transporters in brain and various extracerebral tissues. Concerning this issue, it is described that P-glycoprotein overexpressed in the blood–brain barrier and peripheral organs of subjects with DRE prevents adequate levels of antiseizure medications in plasma and in the brain tissue (see Chap. 6). The relevance of targeting the signaling pathways that upregulate P-glycoprotein in brain capillaries in response to seizure activity is considered a new therapeutic strategy to control DRE (see Chap. 23). However, the validation of this hypothesis is difficult since the administration of transporter inhibitors in patients with DRE does not alter the efficacy to antiseizure medications, even when their plasma and/or concentrations in peripheral tissues are increased. Thus, additional studies are necessary for its confirmation.

Genetic variations are suggested as mechanisms underlying DRE (see Chaps. 10 and 17). Different conditions associated to pharmacogenetic variants can explain therapeutic failure in epilepsy: (a) gene mutations affecting the protein expression of therapeutic targets, (b) genetic variants that increase the adverse effects induced by drugs, and (c) overexpression of genes that alters the pharmacokinetics of the antiseizure drugs. Furthermore, the existence of several haplotypes in the polymorphisms of CYP and transporters provides information that can explain the interindividuals and/or inter-ethnics differences regarding the prevalence of DRE. Like other hypotheses, the proof that genetic variation is responsible for DRE still depends on more studies showing that these variant genes interfere with the action of antiseizure medications in their specific targets.

It is evident that the understanding of DRE as a complex phenomenon leads to visualize innovative strategies, add-on therapies, and antiseizure medications with

complex pharmacology (see Chap. 20). Indeed, it is possible to consider pharmacokinetics characteristics of the antiseizure medications to overcome the resistance condition in epilepsy. These characteristics can include the administration of antiseizure medications under bitherapy regimen but at different dosing intervals; or the co-administration of supplements that may reduce the side effects induced by antiseizure medications (see Chap. 9). Moreover, the use of modern “omics” technologies provides valuable information on the mechanisms involved in the condition of drug resistance in epilepsy and provide novel molecular candidates to be used for the development of new and more effective antiseizure medications (see Chap. 15).

The use of strategies such as intracerebral recordings is necessary to identify the epileptic focus in some patients with DRE candidates to epilepsy surgery (see Chap. 19). It is important to highlight the efficacy of alternative strategies to control DRE, such as ketogenic diet (see Chap. 22), vagus nerve stimulation (see Chap. 24), transcranial magnetic stimulation (see Chap. 25), and transcranial focal electrical stimulation via concentric ring electrodes (see Chap. 26). Actually, the physical exercise can be considered as a complementary strategy to reduce the seizure activity (see Chap. 21) and improve the efficacy of the antiseizure medications (see Chap. 9).

At present, different preclinical models are used to reproduce the complex clinical conditions associated with DRE and look for novel therapeutic strategies. Each experimental model has advantages and limitations (see Chap. 3). Certainly, all of them help to elucidate the underlying mechanisms of the DRE. However, further studies must consider preclinical models that reproduce several clinical conditions of patients with DRE such as age, sex, comorbidities, and treatment with antiseizure medications. An aspect not developed in depth in this book is the current use of Cannabidiol (CBD) as an antiseizure medication for the treatment of DRE, including Dravet syndrome, Lennox Gastaut syndrome, and Tuberous Sclerosis (Devinsky et al. 2018; Thiele et al. 2018; Miller et al. 2020). At present, the therapeutic use of cannabis compounds has begun to be considered to control different pathological conditions associated with eating behavior, lipid metabolism, immune system, addiction, pain, cancer, and epilepsy. However, there are controversial results regarding its effectiveness and safety, whereas their mechanisms by which reduce the seizure activity and the side effects are still not completely evident. Studies support the inhibitory effect of CBD on the most abundant CYP isoforms (Doohan et al. 2021), the glucuronidation system (to a lesser extent), and the excretion transport systems (ABC-transporters) (Auzmendi et al. 2020). These effects can alter the pharmacokinetics of some antiseizure medications. Concerning side effects, studies indicate the risk of hepatotoxicity by chronic use of CBD (Ewing et al. 2019). It is described that the daily administration of CBD at therapeutic doses for 3.5 weeks may induce liver injury characterized by increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT) (Watkins et al. 2021). CBD promotes adipogenesis of human and mouse mesenchymal stem cells via PPAR γ by inducing lipogenesis (Chang et al. 2022). It can also induce anemia, affects fertility (Carvalho et al. 2022), and alters the immune system

with a consequent predisposition to recurrent infections (Martini et al. 2023; Gómez et al. 2021). According to this information, it is essential to carry out pharmacovigilance studies for long-term therapeutic use of CBD with antiseizure medications to detect drug–drug interactions and side effects in patients with DRE (Vázquez et al. 2021).

This book represents an attempt to explain some of the mechanisms underlying the DRE and the complexity of this serious condition. It is evident the necessity of studying with the most modern technologies and across various regions of the world. In addition, a set of shared data coming from the most different groups interested in this question is essential. Great efforts will be needed in the proper organization of research projects, clinical or basic with a translational perspective, to guarantee the emergence of more precise questions. The organization of cross-disciplinary teams with converging perspectives can benefit the study of complex problems such as the DRE and will facilitate the equitable treatment of all issues arising throughout the process leading to more reliable and appropriate answers.

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Chapter 2

Pharmacoresistance in Epilepsy



Daniel San-Juan and Roberto Antonio Cordova Peralta

Abstract Although more than 30 anti-seizure medications are available on the market, epilepsy remains pharmaco-resistant in 30% of patients with focal epilepsy and approximately 10–15% of patients with idiopathic/genetic generalized epilepsy as well. The treatment of these complex patients needs a structured multidisciplinary approach to address the biological, social, and psychological implications. The current chapter is a general overview of epilepsy as a stigma, health, and financial problem and initiatives to change the conditions of patients with epilepsy. Special attention is focused on the effects of pharmacoresistant epilepsy.

Keywords Epilepsy · Pharmacoresistance · Epidemiology · Stigma · Antiseizure medications · International League against Epilepsy · World Health Organization

2.1 Epilepsy

To “take hold of abruptly or to seize” is the meaning of the word Epilepsy, derived from a preposition and an irregular Greek verb (Epilambanein) (Khalil et al. 2020). This term has undergone multiple modifications throughout time and cultures over centuries. In 2005, a Task Force of the International League Against Epilepsy (ILAE) formulated conceptual definitions of seizures and epilepsy. Epilepsy was defined as a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures, and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure (Fisher et al. 2005). However, it was needed to create an operational clinical definition of epilepsy demarcated by any of the following conditions: (1) at least two unprovoked (or reflex) seizures occurring

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>24 h apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; or (3) diagnosis of an epilepsy syndrome. However, this practical definition had implications for the patients diagnosed with epilepsy, including stigma, psychological, social, cognitive, and financial repercussions of relevance as to be built into the conceptual definition of epilepsy (Fisher et al. 2014). The current definition of epilepsy aims to influence early diagnosis and treatment with the purpose to apply disease-modifying treatments that prevent the progression of epilepsy and the onset of comorbidities (Fisher et al. 2014).

2.2 Epilepsy as Stigma

The scientific development of medicine has often clashed with religious beliefs, leading to various flawed concepts that categorize epilepsy as the “sacred disease,” oblivious to the warning by Hippocrates, who tried to convince society that epilepsy was neither more divine nor more sacred than other diseases but had a natural cause like other affections. Regardless of Hippocrates’ wise concepts, absurd beliefs and conceptions multiplied and spread; the patient with epilepsy has been considered to “be possessed,” which, in turn, has resulted in his/her rejection or exclusion not only by society in general but often by their own family. It is well known that, up to this date, patients with epilepsy have been subjected to exorcisms to liberate him/her from “demonic possession,” both in highly developed and underdeveloped countries. Few diseases have been associated with such an accumulation of inconsistent beliefs based on superstition, prejudice, or ignorance as epilepsy. In fact, in several cases, it proves more difficult to control the environment where the epilepsy patient lives than to obtain good seizure control. The stigma persists and is sustained on mystical foundations (Khalil et al. 2020). The clinical manifestations of epilepsy and the different types of epileptic seizures have been described since Babylonian times in the earliest handbooks of medicine on a clay tablet called antashubba, which is Sumerian for “falling disease.” They also had some understanding of prognosis, as the text described several outcomes depending on the type of seizure, including poor outcomes in status epilepticus as well as post-ictal states in other seizure types (Kaculini et al. 2021).

Epilepsy’s long history can be traced back to a 4000-year-old Akkadian tablet found in Mesopotamia; inscribed on it is a clinical description of a subject with “his neck turning left, hands and feet are tense, and his eyes wide open, and from his mouth froth is flowing without him having any consciousness” (Kaculini et al. 2021). This ancient history of epilepsy allowed religious beliefs to be widely spread through the Bible, the Talmud, and the Koran. Paroxysmal episodes are described in the Old Testament and are believed to be episodes of deep sleep (tardemah) that “took hold of Abraham.” It is noteworthy that the word “Tardemah” used in Genesis and translated to Greek is identified as ecstasy, an episode that was frequently experienced by the prophets Isaiah, Daniel, Ezekiel, and Jeremiah. On the contrary, *The*

Book of Revelations in the New Testament contains a detailed description of what is now called “Saint John’s malady,” a disease suffered by the apostle himself with clinical features consistent in auditory manifestations and falls with possible seizures (Mameniškienė et al. 2022), considered by Dostoyevsky as similar to his episodes of ecstatic seizures from the insular origin (Tényi et al. 2016).

Throughout history, this condition has been plagued by mystical misconceptions, including demonic possessions, witchcraft, or divine interventions. This has frequently distorted or even halted any real improvement in its scientific understanding or its social perception. This metaphysical situation is also at the core of the stigma revolving around this condition, some of which still lingers today. In several countries such as Egypt, the stigma of patients with epilepsy was mentioned in an inscription in the temple at Esna, according to which those who were under the “Bas of sorcery” [Bas being the plural of Ba], defined as “a state of existence attained by the diseased in the afterlife,” were restricted access to the temple, with these patients thought to be individuals with epilepsy (Bou Nasif et al. 2021; Griffiths 1970). In China, epilepsy was defined as recurrent attacks in a book titled *The Yellow Emperor’s Classic of Internal Medicine* around 770–221 BC. The Chinese name for epilepsy, Dian, was also used for “insanity,” which indicates the stigma associated with epilepsy (Bou Nasif et al. 2021). One of the Persian terms for epilepsy is “iblisia.” Its origin is theorized to stem from the word “iblis,” which means “the devil,” therefore maintaining the magical beliefs and stigmas accompanying the disease (Vanzan and Paladin 1992).

Many famous historical personalities were theorized to have suffered from epilepsy. For example, Julius Caesar (100–44 BC) was described as having episodes of fainting, shaking, and feeling overpowered by his sensations, possibly due to focal seizures with loss of awareness, and is believed to have had absence seizures during his childhood (Hughes 2004). Joan of Arc (1412–1431), who claimed she could hear the voices of Saints Michael, Catherine, and Margaret, is believed to have had temporal lobe epilepsy with auditory hallucinations associated with elementary or complex visual hallucinations (e.g., a great light or human faces), which are considered ecstatic ictal states by some authors and gave her a sense of divine mission (Nicastro and Picard 2016).

Currently, discrimination against people with epilepsy persists in society, for example, by avoiding friendships with patients with generalized motor seizures (Walther et al. 2022). Stigma is significantly higher in patients with epilepsy and has a detrimental effect on the patient’s quality of life, recovery, and prognosis (Malik et al. 2022). The dissemination of epilepsy knowledge and positive perceptions of epilepsy by increasing self-efficacy throughout a lifetime may reduce self-stigma (Kuramochi et al. 2022). However, in general, the stigma is perceived differently in several countries and cultures and requires specific strategies to reduce the burden (Andersson et al. 2022). Some risk factors related to the stigma are severe epilepsy, unfavorable income, restrictions on daily life, male gender, number of anti-seizure medications (ASMs), older age, chronic epilepsy, and frequency of seizures (Hohmann et al. 2022; Lalatović et al. 2022). Also, stigma in epilepsy is associated with depression, anxiety, being unmarried, and being unemployed (Lee et al. 2022).

Increasing institutional and social awareness to augment social support in patients with epilepsy and providing the patient with positive coping strategies may be an effective strategy for reducing stigma in patients with epilepsy (Karakaş et al. 2022).

2.3 Epilepsy and Pharmacoresistance

The initial treatment of a patient with new-onset epilepsy is usually based on the use of one of the 30 ASMs available on the market. The type of ASM selected varies depending on the type of epilepsy and other clinical variables, including comorbidities. The failure of the first ASM monotherapy is possibly explained by a lack of efficacy or poor tolerability. In the first scenario, it is necessary to exclude inadequate selection of ASMs for the type of epilepsy or epileptic syndrome, lack of adherence to the treatment, drug interactions, or pharmacokinetic factors. Regarding poor tolerability, the patient can develop idiosyncratic adverse effects or adverse effects related to the dose-by-drug interactions (Abou-Khalil 2022; Brigo and Marson 2022). Though patients can fail the initial ASM monotherapy and need to switch to another ASM or add another ASM as an adjuvant to try to become seizure-free (Brigo and Marson 2022). Unfortunately, up to 30% of patients develop drug-resistant epilepsy, defined as failure of adequate trials of two tolerated and appropriately chosen and used ASM schedules (whether as monotherapies or in combination) to achieve sustained seizure (Kwan et al. 2010). Some common risk factors for not achieving 12-month seizure remission included tonic-clonic seizures, focal epilepsy, younger age, and female sex (Brigo and Marson 2022). Regrettably, approximately 10–15% of patients with idiopathic/genetic generalized epilepsy (IGE/GGE) remain drug-resistant (Gesche and Beier 2022).

Focal epilepsy of unknown etiology is the most common type of epilepsy (17.5 patients per 100,000 per year), followed by symptomatic focal epilepsies (focal epilepsies of structural or metabolic origin) and idiopathic generalized epilepsies (3.7%) (Zarrelli et al. 1999). Several hypothesized mechanisms of pharmacoresistant drugs include modifications in receptors and ion channels, changes in drugs transport proteins and enzymes involved in drug metabolism, modifications in neural networks, and intrinsic severity hypotheses; however, isolated, the hypothesized mechanisms of drug-resistance offer only a partial view of the issue (Servilha-Menezes and Garcia-Cairasco 2022). On the contrary, the exact cause of drug resistance in IGE is unknown. From several hypotheses (altered networks, minor cortical lesions, impaired interneurons, and/or drug kinetics) of pharmacoresistant epilepsy in patients with IGEs, the interneuron hypothesis, related to persistent impaired GABAergic signaling of interneurons despite adequate ASM, looks the most promising because it is based on the lack of normalization of intracortical inhibition compared to responders (Badawy et al. 2014); however, not all studies report positive causalities to explain these hypotheses (Gesche and Beier 2022).

2.4 Epilepsy as Health Problem

Epilepsy is one of the most common and severe neurological conditions, with a worldwide prevalence of 6.38 per 1000 people (Alva-Díaz et al. 2021), affecting more than 50 million people of all ages, genders, ethnic backgrounds, and geographic locations worldwide (WHO Epilepsy 2019). Nearly 80% of patients with epilepsy live in low- and middle-income countries, where risk factors such as neuroinfections and suboptimal pre- and perinatal care are more common (Newton and Garcia 2012). Mortality is almost threefold higher in patients with epilepsy compared with the general population (Levira et al. 2017), is placed in the third position among chronic neurologic conditions in terms of disability-adjusted years of life lost (GBD 2015 Neurological Disorders Collaborator Group 2017), and is linked to sudden unexpected deaths related to epilepsy (Thurman et al. 2014). Despite this clinical situation, only 25% of patients with epilepsy in low- and middle-income countries receive appropriate treatment, creating a treatment gap. A lack of adequate treatment also occurs in high-income countries in areas with low levels of health-care access, low health literacy, and poor epilepsy awareness (Kalilani et al. 2019).

The initial international campaign to promote epilepsy as a global medical problem started in 1977 with the collaboration of ILAE, the International Bureau for Epilepsy (IBE), and the World Health Organization (WHO) with the Global Campaign Against Epilepsy, named “Out of the Shadows,” with incremental epilepsy awareness, acceptance, and education in epilepsy. After this global effort, WHO incorporated epilepsy as a priority medical condition in its Mental Health Gap Action Program, which includes evidence-based guidelines for non-specialist primary healthcare providers (Guekht et al. 2021). On May 26, 2015, the collaborative efforts tripartite between ILAE, IBE, and WHO member states—encouraged by China—culminated in the approval of WHA Resolution 68.20, titled “Global Burden of Epilepsy and the Need for Coordinated Action at the Country Level to Address Its Health, Social and Public Knowledge Implications.” Endorsed by all 194 WHO member states, this historic resolution established the recognition of the global burden of epilepsy and the necessity of creating international efforts of WHO member states to take public health actions in epilepsy, multilateral engagement, and a better system of surveillance to monitor the global burden of epilepsy and the financial consequences, including the evaluation of the outcomes (Covanis et al. 2015). In 2018, the first WHO global report on epilepsy “Epilepsy: A Public Health Imperative” was released, considered a global call for action to fight against stigma, modify the legislation, and address the gaps in education, care, and access to safe and affordable ASMs globally (WHO Epilepsy 2019). Finally, on November 2020, the collaboration of these three international entities, the World Federation of Neurology, and the European Federation of Neurological Associations—terminated in the undisputed approval of a WHO resolution to create and implement an Intersectoral Global Action Plan on Epilepsy and Other Neurological Disorders (IGAP) to guide the collaboration during the next two decades, supporting universal health coverage, to address the gaps in the promotion of physical and mental health,

and prevention, early detection, care, treatment, and rehabilitation, as well as social, economic, educational, and inclusion needs of persons and families living with epilepsy and other neurological disorders, and the ongoing need for research to improve prevention, early detection, treatment, care, and rehabilitation, including treatment options with the potential to cure epilepsy and other neurological disorders (Guekht et al. 2021). This is an unprecedented opportunity to guarantee that epilepsy is placed high on local governmental agendas worldwide, establish synergic collaboration in healthcare with other neurological comorbidities, use epilepsy as an entry point to improve the health system across conditions affecting the nervous system, continue decreasing the stigma, and incorporate the patient with epilepsy into society without any discrimination (Guekht et al. 2021). In May 2022, during the [75th World Health Assembly](#) in Geneva, Switzerland, four international organizations and 116 member states spoke in support of the IGAP. On May 27, 2022, WHO Member States [unanimously approved the plan](#) with the 90-80-70 cascade target for epilepsy: 90% of all people with epilepsy are aware of their diagnosis as a treatable brain disorder; 80% of people with epilepsy have access to appropriate, affordable, and safe ASMs; and 70% of people with epilepsy on treatment achieve adequate seizure control (“Draft Intersectoral Global Action Plan on Epilepsy and Other Neurological Disorders (IGAP) // ILAE,” 2022).

2.5 Burden of Pharmacoresistant Epilepsy

Epilepsy, like any other neurological condition, has direct costs for the patient and society due to the expense of healthcare and other social services for evaluation, treatment, and rehabilitation. It also involves indirect costs for disabling complications and high rates of mortality, as well as limitations to appropriate education, employment, and other financial activities related to the stigma around the disease. These situations limit the ability to obtain a normal life with adequate quality of life. Nearly two-thirds of patients with epilepsy become seizure-free after the use of adequately chosen ASMs, with an estimated cost of US\$5 per year (WHO Epilepsy 2019). It is well known that the treatment of epilepsy is cost-effective, reducing the financial burden of the condition (Megiddo et al. 2016). Unfortunately, there is a treatment gap for epilepsy in approximately 50% of patients with epilepsy from countries of low-middle income, which do not receive adequate ASMs (Radhakrishnan 2009).

A recent review included 101 cost-of-illness studies that analyzed the direct healthcare cost database, mainly from North America or Western Europe, and 13 studies from indirect cost databases, which found that the total cost of epilepsy was 119.27 billion with a huge disparity in the mean annual cost per person with epilepsy in 2019 in low-income countries (US\$204) compared with high-income countries (US\$11,432) (Begley et al. 2022). However, epilepsy’s direct costs are below those of other common medical conditions. For example, healthcare costs for epilepsy in the United Kingdom in 2019 were \$955.86 million, compared to dementia

at \$1.57 billion, cancer at \$3.96 billion, coronary heart disease at \$2.16 billion, and stroke at \$1.65 billion (Wimo et al. 2017).

The direct cost of epilepsy related to the use of ASMs accounts for 40–78%, followed by hospitalizations (Gao et al. 2015; Hong et al. 2009). Gao Lan et al., in 141 Chinese patients with epilepsy, reported that the cost of ASMs was US\$394.53. 53.9% were treated in polytherapy and 83% with at least one type of new-generation ASMs. Guekht et al. conducted a study on 738 adolescents and adults with epilepsy seen in the ambulatory services of a city hospital in Moscow and reported a median cost of ASMs of €643 (IQR 288–1866; range 0–9960), ranging from €782 in newly diagnosed patients to €3777 in patients with drug-resistant epilepsy (Guekht et al. 2016). Fallah et al. (2016) performed a cost-utility analysis, from a third-party payer perspective, for children with Tuberous Sclerosis Complex and drug-resistant epilepsy that had failed to improve with 2 ASMs and found that the addition of a third ASMs (US\$6600 for a gain of 4.14 quality-adjusted life years) was the most cost-effective treatment strategy compared to resective epilepsy surgery, vagal nerve stimulation (VNS) implantation, or ketogenic diet (Fallah et al. 2016).

The clinical benefit of epilepsy surgery in well-defined patients with focal temporal lobe pharmaco-resistant epilepsy has been previously tested (Engel et al. 2012; Wiebe et al. 2001), with a long-term reduction in costs associated with epilepsy, particularly in patients who become seizure-free (–32%) at 2 years of follow-up due to less use of ASMs and inpatient care (Langfitt et al. 2007). Unfortunately, it is estimated that only 5–10% finally underwent epilepsy surgery for multiple reasons (Jetté et al. 2016). Several studies from different countries with heterogeneous healthcare systems show repeatedly that patients receiving multiple ASMs are more expensive than epilepsy surgery. A recent systematic review found that surgical treatment is cost-effective compared to medical treatment, especially in the long term (Kitschen et al. 2022).

Many patients who are not candidates for resective epilepsy surgery but still receive multiple ASMs underwent additional therapies such as VNS, with an average total cost estimated at EUR 7703.59 in year 1 and EUR 7108.38 in year 2 following VNS implantation. A review found that the average direct costs of VNS treatment of patients with pharmacoresistant epilepsy over the last 18 years varied between countries and ranged from €24790.43 in the United States to €64.84 in the United Kingdom (Kopciuch et al. 2019).

2.6 Epilepsy Care

Globally, there are multiple and relevant gaps in the delivery of healthcare for patients with epilepsy around the world. The absence of adequate diagnosis and care prevents patients from achieving their full potential and poses a significant burden to countries' economic, health, and social systems. Low- and middle-income countries are excessively impacted by this situation. The IGAP (2022–2031), adopted in 2022 by the World Health Assembly, offers the opportunity to conduct a

multisectoral and international approach to improve the diagnosis and treatment of epilepsy worldwide. The adaptation of new policies depends on the setting and its priorities to obtain the outcomes necessary to improve the lives of patients with epilepsy. For example, create new models of care, provide ASMs, funding, and allocation of resources; create new laws or rules to reduce stigma and discrimination; increase research in the field; create novel systems for improving the quality of care; and so on (Guekht et al. 2021).

Country-level initiatives for reacting to the epilepsy treatment gap show that significant gains can be realized. In the “Fight against epilepsy” initiative of the Ministry of Health of Ghana, in partnership with WHO, treatment for epilepsy in the primary care sector improved from 15% to 38% after training primary health-care workers and community volunteers, increasing awareness, and strengthening monitoring and evaluation (WHO Epilepsy Ghana, 2018). In the Myanmar Epilepsy Initiative, these partnerships improved community coverage from 2% at baseline to 47% (Myanmar epilepsy initiative: piloting the WHO program on reducing the epilepsy treatment gap 2013–2017, 2018).

2.7 Conclusion

Epilepsy is a very complex neurological disease that affects more than 50 million people worldwide with a millenary history of stigma and discrimination that influence many aspects of the lives of patients living with this condition. The treatment gap for epilepsy is not only limited to ASMs, and the drug-resistance is a real problem with high costs and side effects for many and diverse patients. Currently, epilepsy is considered a public health priority by WHO member states because there are significant gaps in the provision of care for epilepsy around the world, and new global action plans, supported by international organizations, are the next challenges for the next two decades.

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Chapter 3

Experimental Models for the Study of Drug-Resistant Epilepsy



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Abstract Drug-resistant epilepsy is a major health problem that has not diminished despite the existence of new antiepileptic drugs. The use of appropriate experimental models that reproduce the characteristics of drug-resistant epilepsy can provide the underlying causes of this disorder as well as the search for therapeutic strategies that are effective for its control. This chapter describes the preclinical models currently used to study drug-resistant epilepsy and their advantages and limitations.

Keywords Drug-resistant epilepsy · Preclinical models · In vitro models · In vivo models · Human brain tissue

3.1 Introduction

Experimental models make it possible to clarify the mechanisms underlying disorders such as epilepsy and facilitate the development of new therapeutic strategies for its control. However, due to the complexity of drug resistance in epilepsy, it is difficult to model this condition. This chapter focuses on describing the currently available models of drug-resistant epilepsy, as well as discussing their advantages and limitations.

The first model of drug-resistant seizures was reported in 1953 by Brown et al. These authors analyzed the in vivo effect of anticonvulsant drugs in mice in the minimum threshold model of electroshock and in the model of psychomotor seizures following the application of 6-Hz. The results obtained showed that in both models, the animals did not respond to phenytoin and that, on the contrary, they

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presented convulsive seizures of greater severity and duration (Brown et al. 1953). Subsequently, Barton et al. extended these findings and characterized drug resistance in psychomotor seizures by corneal electrical stimulation of 6 Hz (Barton et al. 2001). Currently, there are different in vitro and in vivo models that reproduce the characteristics of drug-resistant seizures and epilepsy. The different models and their specific characteristics, advantages, and limitations are presented below.

3.2 In Vitro Models

In vitro models make it possible to reproduce some of the conditions of drug-resistant epileptic activity in a controlled manner.

3.2.1 Cell Cultures

Cell cultures used as in vitro models of drug-resistant epilepsy include the use of brain cell lines, such as endothelial cells and immortalized neurons, among others (Cecchelli et al. 2007). Cultures can be of a single cell type (monocultures) or cocultures of several different types of cells, such as endothelial cells, pericytes, and astrocytes.

Cell cultures represent simple biological systems useful for the search for new therapeutic strategies for the control of drug-resistant epilepsy (Campos et al. 2018; Wong 2011). In this regard, it is known that cell cultures allow the investigation of drugs that cross the blood–brain barrier (BBB) despite the overexpression of transporters in this structure and act at the neuronal level (Luna-Tortós et al. 2008; Zhang et al. 2012). Likewise, the use of cell cultures makes it possible to investigate experimental strategies that favor the passage of drugs into the brain parenchyma (Bohosova et al. 2021).

Cell culture use optimizes experimentation time and resources and involves experimental procedures that are easily reproducible. Additionally, the bioethical requirements for its use are lower than those required for in vivo models (Hartung and Daston 2009). Regarding their limitations, it is important to consider that cell cultures present artificial conditions that do not reproduce the complexity of a complete living being (Wahab et al. 2010). Another limitation is that cell culture use is restricted to short-term conditions because in vitro cells lose their properties and dedifferentiate after a certain time (Abbott et al. 2006; Campos et al. 2018; Cecchelli et al. 2007; Roux and Couraud 2005).

3.2.2 Brain Slices with Cortical Dysplasia Exposed to 4-Aminopyridine

Exposure of brain slices to 4-aminopyridine (4-AP, a K⁺ channel blocker that induces neuronal hyperexcitability) is used to evoke epileptiform activity in neurons in structures such as the hippocampus and cerebral cortex (Galvan et al. 1982; Pasantes-Morales and Arzate 1981). On the other hand, exposure of pregnant rats to methylazoxymethane acetate (MAM) produces cortical molecular and cellular alterations in offspring similar to those of cortical dysplasia, a condition that favors drug-resistant epilepsy (Smyth et al. 2002). Exposure of brain slices from rats previously exposed to MAM to 4-AP reproduces drug resistance since epileptiform activity is not modified by exposure to ASMs such as valproic acid, ethosuximide, or lamotrigine (Smyth et al. 2002).

This in vitro model has the advantage of being low cost and quick to perform. Its main limitation is that brain slices do not have the connectivity and integrity of a complete brain. Additionally, brain sections from this model do not display the spontaneous ictal activity characteristic of epilepsy.

3.2.3 In Vitro Study of Brain Tissue from Patients with Drug-Resistant Epilepsy

Epilepsy surgery is a strategy used to treat patients with epilepsy who do not respond adequately to ASMs. The in vitro study of brain tissue obtained from epilepsy surgery allows a more direct approach to the conditions related to drug-resistant epilepsy in patients and avoids several limitations inherent in experimental models (Morris 2021). The brain tissue of patients with drug-resistant epilepsy obtained during epilepsy surgery can be used for different evaluations, such as in vitro neurotransmitter release, receptor binding assays, in vitro electrophysiological recordings, cell culture, and estimation of molecular, genomic, and epigenetic changes, among others. The manipulation of the brain tissue will depend on the experimental procedure that will be used. One limitation of the use of brain tissue from patients with epilepsy is the difficulty of obtaining brain tissue from healthy subjects for comparison. This situation can be resolved in different ways. One is to analyze brain tissue without epileptiform activity obtained from areas surrounding the epileptogenic focus. An alternate possibility is to use brain tissue from deceased subjects with no neurological history as control tissue (Huberfeld et al. 2015; Kirchner et al. 2020; Rocha et al. 2007, 2012).

An advantage of this preparation is that the correlation of the results obtained with the clinical variables of each patient allows to identify clinical factors that influence the expression of drug-resistant epilepsy (Loeb 2010). Regarding the limitations that the use of human brain tissue entails, there is the damage that is induced during its resection, as well as the time that elapses from its handling to its

storage or use (Morris 2021). In addition, studies are required to comply with complex bioethical requirements.

3.3 In Vivo Models of Drug-Resistant Seizures

Appropriate in vivo models exist to identify candidate drugs for the control of drug-resistant epilepsy in the early phases. These models are used to reproduce some conditions associated with drug resistance and thus study the mechanisms involved in it, as well as test the effects of new ASMs. One limitation of these models is that they are acute models and do not reproduce spontaneous epileptic activity.

3.3.1 *Caenorhabditis Elegans*

Caenorhabditis elegans (*C. elegans*) is a one millimeter long, transparent nematode (Locke et al. 2009). This nematode self-fertilizes, and its development takes approximately 3 days (Girard et al. 2007; Locke et al. 2009). *C. elegans* has a relatively simple nervous system. However, it contains the function of ion channels and neurotransmitters in the nervous system of more evolved animals, such as mammals (Jin et al. 1999; Richmond and Jorgensen 1999).

C. elegans is a model in which it is possible to study neuronal hyperexcitability and epileptiform activity associated with genetic mutations. This nematode is considered an economic model to analyze the mechanisms related to the development of drug resistance in epilepsy (Baraban 2007, 2009). Susceptibility to proconvulsant drugs such as pentylenetetrazole (PTZ) in the presence of mutations has been evaluated with this model (Williams et al. 2004). However, this model has the limitation that it does not allow the evaluation of spontaneous epilepsy.

3.3.2 *Zebrafish*

Zebrafish or *Danio rerio* is a small freshwater fish that belongs to the *Cyprinidae* family. Zebrafish is an easy-to-handle vertebrate; it has a fast reproduction rate, in addition to having organs similar to mammals, including the brain and spinal cord (Rupp et al. 1996; Wullimann and Mueller 2004; Wullimann 2009). Zebrafish has been used to induce seizures by exposure to chemical agents such as PTZ (Afrikanova et al. 2013; Baraban et al., 2007) or allylglycine (Leclercq et al. 2015). It has also been used to analyze the influence of genetic mutations in epilepsy (Chege et al. 2011; Grone and Baraban 2015; Zhang et al. 2015).

Currently, zebrafish are considered a suitable experimental model for the study of drug-resistant seizures (Baxendale et al. 2012; Leonard and Randall 2005;

Stewart et al. 2012). Its use is economical and does not require complex installations. However, the identification of recurrent and/or spontaneous ictal events is not easily achieved (Stables et al. 2002; Stewart et al. 2012).

3.4 Induction of Drug-Resistant Seizures by Repeated Administration of Proconvulsant Drugs

Repeated and frequent induction of severe seizures is proposed to facilitate the phenotype of drug resistance in epilepsy (Rogawski and Johnson 2008). Currently, an experimental strategy that models this situation is the repeated and short-term induction of generalized and severe seizures by the repeated administration of proconvulsant drugs. In this regard, the repeated induction of clonic–tonic seizures by daily administration of PTZ for at least 7 days has been shown to result in the induction of phenytoin-resistant seizures (Auzmendi et al. 2013). Similarly, the repeated induction of generalized seizures by repeated administration of 3-mercaptopropionic acid (3-MP), a hydrophilic thiol (Salgado et al. 2015) that increases glutamate levels by inhibiting the enzyme glutamic acid decarboxylase (GAD) (Lamar 1970), results in generalized clonic–tonic seizures resistant to phenytoin and phenobarbital but responsive to diazepam, carbamazepine, and levetiracetam (Enrique et al. 2017; Pérez-Pérez et al., 2021). Additionally, throughout the repeated administration of 3-MP, the animals progressively present status epilepticus (convulsive seizures of ≥ 5 min), which correlates with the establishment of drug resistance (Frías-Soria et al. 2021). Repeated administration of PTZ or 3-MP also induces overexpression of P-glycoprotein in neurons, astrocytes, and endothelial cells, an effect that is associated with the development of the drug-resistant phenotype (Auzmendi et al. 2013; Enrique et al. 2017; Lazarowski et al. 2004; Rosillo-De La Torre et al. 2015).

An advantage of inducing drug-resistant seizures with repeated administration of proconvulsant drugs is the short experimental time required for their induction. On the other hand, one limitation is that the experimental subjects do not present spontaneous seizures (Pérez-Pérez et al., 2021; Frías-Soria et al. 2021).

3.5 Chemical Kindling and Drug Resistance

Chemical kindling consists of inducing epileptogenesis by the repeated administration of initially subconvulsant doses of drugs such as PTZ (Mason and Cooper 1972). Chemical kindling represents a good strategy for obtaining animals with drug-resistant seizures.

Coriaria lactone, a drug extracted from the *Loranthus Coriaria sinica Maxim* plant (Zhou et al. 2006), induces proconvulsant effects by activating NMDA

receptors and increasing glutamate release (Wang et al. 2003). Chemical kindling from repeated administration of Coriara lactone culminates in clonic–tonic seizures (Gilbert 2006) resistant to carbamazepine, phenytoin, and valproate and overexpression of ASM transporters in the BBB (Wang et al. 2003). In contrast, lamotrigine and topiramate retain their anticonvulsant effect in this model (Wang-Tilz et al. 2006).

During the development of chemical kindling by PTZ, repeated administration of lamotrigine, a voltage-gated sodium channel blocker, does not arrest epileptogenesis but results in the development of resistance to different ASMs (lamotrigine, levetiracetam, carbamazepine, zonisamide, gabapentin, pregabalin, phenytoin, and topiramate) but not valproate (Kumar and Goel 2020; Singh et al. 2014).

The advantage of chemical kindling is its easy implementation (Singh et al. 2021). Among its limitations is the absence of spontaneous seizures, since the administration of proconvulsant agents is required to obtain the manifestation of seizures.

3.6 Electrical Kindling and Drug Resistance

Electric kindling was described in 1967 (Goddard 1967), and it has been used in different animal species, such as baboons (Wada et al. 1975), rodents (Boldt et al. 2021) and cats (Valdés-Cruz et al. 2019). Electrical kindling results from the repeated application of an initially subthreshold electrical stimulus in areas of the brain such as the hippocampus and the cerebral amygdala, among others, which progressively leads to the expression of kindled-type epileptic seizures (Goddard et al. 1969).

Using electrical kindling, it has been found that once the kindled state was reached, animals presented different responses to phenytoin. Twenty percent of kindled animals are drug resistant and characterized by a more rapid development of epileptogenesis as well as high extracellular levels of glutamate in the interictal period (Luna et al. 2011). This effect is similar to that observed in patients with drug-resistant temporal lobe epilepsy (Cavus et al. 2005; During and Spencer 1993). Forty percent of kindled animals are responsive to phenytoin, while 50% present variable responses (Löscher and Rundfeldt 1991; Töllner et al. 2011).

In rats, repeated administration of lamotrigine throughout the development of electrical kindling of the amygdala promotes resistance to lamotrigine and carbamazepine, while sodium valproate maintains its anticonvulsant effect (Postma et al. 2000; Srivastava and White 2013).

A limitation of electrical kindling is that it requires special surgical skills, access to a stereotaxic apparatus, an electrical stimulator, and isolation of the implanted rats. It is a chronic experimental procedure, and the animals do not present spontaneous epileptic seizures (Löscher et al. 1993).

3.7 Corneal Electric Kindling

The 6-Hz model was initially designed for the evaluation of “psychomotor seizures” and complex partial seizures (Toman et al. 1952; Toman 1951). This model was discontinued shortly after its description due to its lack of sensitivity to phenytoin. In 2001, Barton et al. demonstrated that 6-Hz corneal electrical stimulation represents a useful model for the study of resistance to ASM, such as phenytoin, lamotrigine, and topiramate (Barton et al. 2001).

The model is carried out in rats or mice and consists of inducing convulsive seizures by means of application of electrical stimulation (a train of rectangular pulses of 0.2 ms duration at 6 Hz with intensities depending on the animal species, for 3 s), through corneal electrodes (Metcalf et al. 2017). It requires the administration of ophthalmic anesthesia before the placement of the corneal electrodes (Tanaka and Mishima 1953). This model mimics partial seizures, which are characterized by forelimb clonus (Barton et al. 2001).

The seizure activity induced by this model is resistant to the effects of clonazepam, valproate, carbamazepine, and levetiracetam (Barton et al. 2001; Leclercq et al. 2015). Among the advantages of this model is its easy induction, its low cost, and the evaluation of ASMs in a relatively short time (Löscher and Schmidt 2011). One drawback is that animals do not have spontaneous seizures (Barton et al. 2001).

3.8 Models of Drug-Resistant Epilepsy

There are more complex experimental models in which animals display spontaneous epileptic activity that is drug resistant. Some of them are described below.

3.8.1 *Drug-Resistant Epilepsy Secondary to Status Epilepticus Due to Lithium-Pilocarpine*

The induction of status epilepticus (SE) by pilocarpine results in epileptogenesis, temporal lobe epilepsy, and eventually drug-resistant epilepsy (Löscher 2017). It is induced by intraperitoneal administration of pilocarpine, a muscarinic agonist, in rodents pretreated with lithium. Rodents develop automatisms, limbic seizures, and the establishment of SE (Leite et al. 1990; Cavalheiro et al. 1991). Most of the animals that survive this procedure develop spontaneous epileptic seizures some days after SE (Curia et al. 2008). In 60% of animals, spontaneous epileptic seizures can be controlled with ASMs such as levetiracetam, phenobarbital, phenytoin, valproate, and topiramate. In contrast, 40% of the animals do not respond to drug treatment, so their seizures are considered drug resistant (Löscher 2007).

This model allows the study of drug resistance in the chronic phase during the occurrence of spontaneous seizures (Löscher 2017). Among the limitations of this model is the high mortality as well as the long time required to obtain animals with a drug-resistant phenotype.

3.8.2 Kainic Acid and Drug-Resistant Epilepsy

Kainic acid (AK) is a compound naturally extracted from marine algae that has a high affinity for kainite-type glutamatergic receptors. AK is a molecule with neurotoxic effects (Johnston et al. 1974; Olney et al. 1974) that activates excitatory pathways. Its administration induces SE and, in the long term, results in spontaneous epileptic seizures and damage to structures such as the hippocampus, amygdala, piriform cortex, thalamus, striatum, and cerebral cortex (Ben-Ari 1985; Medvedev et al. 2000; Sperk et al. 1983; Mizuno et al. 2021). In this model, 30% of the animals have epilepsy resistant to lamotrigine, valproate, ethosuxamide, gabapentin, levetiracetam, and felbamate (Thomson et al. 2016; West et al. 2016).

Klein et al. found that intrahippocampal administration of AK in mice induces nonconvulsive focal seizures that are resistant to carbamazepine and phenytoin, and the effect of valproate and levetiracetam is attenuated (Klein et al. 2015).

Similar to the lithium-pilocarpine model, the AK model allows investigation of drug resistance in animals with spontaneous epileptic seizures (Löscher 2017). Its limitations are the high mortality and the long experimentation time required to obtain results.

3.8.3 Models of Drug-Resistant Posttraumatic Epilepsy

Traumatic brain injury (TBI) is a global public health problem with high rates of morbidity, disability, and mortality (Jiang et al. 2019). Among the consequences of TBI are the development of seizures and posttraumatic epilepsy (Dikmen et al. 2003). The incidence of developing drug-resistant epilepsy in subjects with posttraumatic epilepsy ranges from 15% to 34% (Kalilani et al. 2018). In drug-resistant posttraumatic epilepsy, carbamazepine and carisbamate have been shown to be ineffective, whereas the anticonvulsant effects of valproate are preserved (Eastman et al. 2011; Barker-Haliski and Steve White 2020).

Despite the large number of animal models available to investigate the molecular and cellular mechanisms of posttraumatic epilepsy, these are not used to investigate drug resistance in the preclinical setting due to the low number of animals that develop epilepsy several months after trauma (Pitkänen et al. 2011).

3.8.4 Canines with Drug-Resistant Epilepsy

Epilepsy is a common pathology in dogs, with an estimated prevalence of 0.6–0.75% (Berendt et al. 2015). Epilepsy in canines is considered to represent a natural model for the study of biomarkers related to epilepsy and its drug resistance (Potschka 2013), as well as the evaluation of new therapeutic strategies (McGrath et al. 2019; Fischer et al. 2022; Potschka et al. 2022).

The manifestation of epileptic activity in canines is very similar to that observed in humans. Canines may present focal seizures (with or without impaired or altered consciousness) and generalized tonic–clonic seizures (Leppik et al. 2009), along with observed interictal and ictal EEG patterns similar to those in humans (Berendt et al. 1999).

One limitation of this model is the complexity of its evaluation by invasive procedures. Additionally, the approval of ethics committees specialized in canine trials is needed. Logistically, it is difficult to obtain an adequate number of dogs to obtain significant results (Patterson 2014). Another limitation of this model is that ASMs have shorter half-lives in canines than in humans (Löscher et al. 1985), making it difficult to maintain constant plasma levels. In addition, there are few ASMs approved for canines (phenobarbital, lamotrigine, and vigabatrin), which have different pharmacokinetics than human and can eventually induce cardiotoxic and neurotoxic effects (Gibson et al. 1990).

3.9 Models of Drug-Resistant Epilepsy Due to Genetic Alterations

The mutation of specific genes in rodents allows the reproduction of some types of drug-resistant epilepsy. The use of this strategy facilitates the search for therapeutic strategies to control said disorder.

Mutations in the SCN1A gene, which encodes the Nav1.1 sodium channel, are associated with the development of epilepsy, such as Dravet syndrome (Scheffer and Nabbout 2019). Using the CRISPR/Cas9 gene editing technique, the SCN1A gene mutation is induced in mice and results in spontaneous seizures (Higurashi et al. 2022). Mutations in this gene change the proteomic profile of elements involved in neurotransmitter dynamics, receptor and ion channel function, synaptic plasticity, astrogliosis, neoangiogenesis, and nitric oxide signaling. In addition, rodents with SCN1A mutations do not achieve adequate pharmacological control of seizures or improvement in associated behavioral comorbidities (Scheffer and Nabbout 2019; Wong et al. 2021).

Patients with mutations in the MECP2 gene, which participates in the expression of methyl-CpG-binding for the development of the nervous system, are highly susceptible to presenting drug-resistant epilepsy (Marafi et al. 2019). There are currently several mouse models that express different types of MECP2 gene

mutations (Katz et al. 2012). Mice exhibit generally short-lived spontaneous discharges that correlate with behavioral arrest and changes that correlate with absence epilepsy (D'Cruz et al. 2010; Wither et al. 2018). The type of epileptic activity that occurs with deletions in the MECP2 gene is resistant to phenytoin, carbamazepine, and levetiracetam (Wither et al. 2018).

The cyclin-dependent kinase 5 (CDKL5) gene is involved in the structural development of the dendritic spine and synaptic activity in excitatory neurons (Ricciardi et al. 2012). The CDKL5 gene mutation is one of the most common features in the diagnosis of epilepsy (Symonds et al. 2019). Ninety-eight percent of patients with the CDKL5 gene mutation can present infantile spasms, myoclonus, and prolonged generalized tonic-clonic seizures (Fehr et al. 2016), which in the long term become drug resistant to various ASMs, such as lamotrigine, carbamazepine, and phenobarbital (Müller et al. 2016). Currently, there are models with CDKL5 mutations that show hyperexcitability (Okuda et al. 2017), visual impairments (Mazziotti et al. 2017), and memory and behavior disorders (Wang et al. 2012). However, they do not present spontaneous ictal or interictal activity, probably due to compensatory mechanisms for the loss of function of CDKL5 (Okuda et al. 2017).

Currently, animal models with genetic modifications are rarely used to assess drug resistance in epilepsy. In the future, these models could contribute new perspectives for the development of new treatment options for patients with drug-resistant epilepsy.

3.10 Novel Approaches to Assess Drug-Resistant epilepsy in Animal Models

In drug-resistant epilepsy, it is important to consider clinical aspects that must be reproduced in preclinical models to facilitate the search for strategies to control it. At the clinical level, drug-resistant epilepsy is expressed in different patterns: epilepsy that is drug-resistant from onset, epilepsy whose drug-resistance status fluctuates with periods of responsiveness to ASMs, drug-resistant epilepsy that is expressed after an initial period of responsiveness to ASMs, or the initial condition of drug resistance that disappears over time.

Some animal models allow evaluation of some of these different patterns of drug-resistant epilepsy. In this regard, the induction of epilepsy secondary to SE by lithium-pilocarpine facilitates the evaluation of animals with epilepsy and different responses to ASMs, i.e., animals with drug-sensitive, drug-resistant and variable-response (Lemos and Cavalheiro 1991). Drug-resistant epilepsy that is expressed after an initial period of responsiveness to ASMs may result from desensitization or downregulation of the therapeutic target as a consequence of repeated administration of such drugs. To reproduce this condition, models can be used, such as the kindling method associated with the repeated administration of ASMs throughout their

development, which results in drug resistance (Löscher and Schmidt 2011; De Petrocellis et al. 2011).

On the other hand, optogenetics is characterized by the optical control of cell activity through artificial incorporation by viral transduction of light-sensitive proteins into cell membranes. Optogenetics makes it possible to control cell activity in specific brain areas (Osawa and Tominaga 2021; Kovac and Walker 2014; Krook-Magnuson et al. 2013) and is considered an experimental strategy to reduce neuronal overactivation and prevent epilepsy drug resistance (Xu et al. 2022). However, optogenetic strategies can also be used to induce overactivation of excitatory cells in specific brain areas, generate neuronal hyperexcitability and epileptiform activity, and facilitate drug resistance.

3.11 Epilepsy Therapy Screening Program: Advantages and Limitations for the Detection of Therapies for Drug-Resistant Epilepsy

The main use of experimental models of drug-resistant epilepsy focuses on the identification of drugs that can be applied at the clinical level to treat this disorder. In this regard, the National Institute of Neurological Disorders and Stroke/National Institutes of Health developed the Anticonvulsant Screening Program. This program involves the use of rodent models and is divided into several phases. The initial phase is identification and involves the use of acute seizure models such as the electroconvulsive maximal shock model (generalized tonic-clonic seizure model) and the 6 Hz electrical stimulation model at a current of 44 mA (generalized tonic-clonic seizure model) to induce focal seizures. The identification phase also involves the use of models associated with cerebral hyperexcitability secondary to recurrent seizures, such as the corneal kindling model and the use of brain slices from animals previously exposed to SE by KA. In this phase, behavioral evaluations are also carried out with the purpose of identifying the therapeutic index based on the efficacy and toxicity of the drugs evaluated. Drugs that show efficacy in the identification phase are then evaluated in the differentiation phase. In the latter, the following models of chronic epileptic activity are used: the model of temporal lobe epilepsy in mice induced by intrahippocampal administration of KA; the rat model of amygdala kindling resistant to lamotrigine; and the model of chronic epileptic activity secondary to the induction of SE by KA in rats. The Theiler model of viral encephalitis in mice, which reproduces epilepsy secondary to viral infections, has been incorporated in the differentiation phase (Kehne et al. 2017).

Based on this program, at least 9 ASMs have been identified. However, a limitation of this program is that only models of temporal lobe epilepsy are involved in the differentiation phase. In addition, the program does not consider the different populations that are generated with the models in relation to their responses to ASMs, i.e., sensitive, resistant, and variable response animals. The Anticonvulsant

Screening Program battery of tests does not consider drug resistance to the new ASMs or clinical factors such as gender, age, and the presence of comorbidities such as anxiety and depression. Based on the above, it is clear that there is a need to redesign the Anticonvulsant Screening Program to provide a more complete evaluation of the drugs that are candidates for ASM for the control of drug-resistant epilepsy.

3.12 Conclusions

It is important to note that there are a great variety of types of epilepsy. Therefore, the study of drug-resistant epilepsy has become very complex. The great diversity of types of epilepsy indicates the complexity of this human condition. Such diversity stems from the different etiologies, the mechanisms underlying epileptogenesis, the neuronal circuits involved, the occurrence of anatomical, and/or functional alterations, etc. Therefore, it is very likely that all these differences are a determining part of the bases of resistance to drug treatment, making the study of the drug-resistant epilepsy also quite complex. It is necessary to use experimental models that reproduce the characteristics of each type of drug-resistant epilepsy and include the possible influence of clinical conditions such as sex, age, duration of the disorder, its severity, and the presence of comorbidities. Collaboration between various groups of researchers and the establishment of networks could facilitate obtaining results in animal models that can be applied at the clinical level to solve the problem of drug resistance in epilepsy.

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Chapter 4

On Complexity and Emergence: Linking the Hypotheses of Pharmacoresistance in Epilepsy



Gabriel Servilha-Menezes, Alan Talevi, and Norberto Garcia-Cairasco

Abstract Epilepsy is a complex disease mainly defined by the susceptibility to spontaneous recurrent seizures but is also often associated with complex comorbid conditions. Despite the introduction of dozens of new antiseizure medications (ASMs), pharmacoresistance, which affects up to 30% of patients with epilepsy (PWE), remains a significant challenge for PWE and epileptologists. To better discuss the numerous proposed hypotheses of pharmacoresistance and the association of their underlying mechanisms, we have categorized them into *bottom-up*, *drug-level*, and *top-down* hypotheses. These categories aim to reflect the brain's hierarchical “network within networks” organization, a concept that guided our discussion on how a complex systems approach, based on systems biology and systems pharmacology, may help solve the complex problem posed by pharmacoresistant epilepsy. Finally, we strongly propose the need for multi-institutional, transnational collaborative efforts that imply urgent paradigm shifts, the sharing of scientific and technological advances, and the construction and use of open algorithms and data repositories/platforms.

Keywords Systems biology · Systems pharmacology · Comorbidities · Complexity · Emergent properties · Epilepsy · Pharmacoresistance mechanisms

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4.1 Introduction

Despite the availability of dozens of antiseizure medications (ASMs), pharmacoresistance remains a significant challenge in patients with epilepsy (PWE) and a puzzling problem for epileptology (Servilha-Menezes and Garcia-Cairasco 2022). The term epilepsy refers to a diverse set of specific conditions: diseases with varying etiologies, risk factors, and clinical manifestations, but linked by a shared distinctive feature: neuronal hypersynchrony and hyperexcitability and an enhanced probability of generating spontaneous recurrent seizures (SRS) (Fisher et al. 2014; Thurman et al. 2011). In summary, epilepsy is a complex disease and, in most cases, the result of the interaction of a vast array of genetic and environmental factors rather than a single point mutation in a specific gene (Koeleman 2018; Thomas and Berkovic 2014). Moreover, they are often associated with complex comorbid conditions, such as anxiety, depression, schizophrenia, or even arthritis (Keezer et al. 2016). Due to the multifactorial nature of epilepsy, to solve the problem of drug-resistant epilepsy (DRE), the current paradigm needs to change towards a complex systems approach (Garcia-Cairasco 2009; Garcia-Cairasco et al. 2021; Margineanu 2012; Talevi 2022; Tejada et al. 2013). To achieve this, the simplistic and outdated view that epilepsy is an imbalance between inhibition and excitation in the brain needs to be revised, and epilepsy must be understood as a multiscale “network within networks” problem.

Although pharmacoresistance is a common condition affecting 25–30% of patients with epilepsy, its causes are not completely understood (Kalilani et al. 2018; Sultana et al. 2021). Over the years, many different mechanisms of drug resistance have been described, leading to several hypotheses. However, there is a growing consensus that each hypothesis offers only a limited explanation of much larger phenomena (Löscher et al. 2020; Pérez-Pérez et al. 2021; Servilha-Menezes and Garcia-Cairasco 2022; Tang et al. 2017). In fact, the supporting evidence for these hypotheses presents many converging aspects that link them into what could be a more complex and complete set of mechanisms. Thus, multidrug pharmacoresistance is likely not the consequence of a single set of alterations but rather the sum of many different alterations spanning different hypotheses, combined in different ways for each individual (Servilha-Menezes and Garcia-Cairasco 2022), and this, for sure, is the support for so-called precision medicine.

4.2 Emergence: Linking Multiple Hypotheses of DRE

Understanding the different alterations found in patients and animal models of DRE and how they connect may lead to the discovery of new mechanisms and help develop new therapeutic approaches. To better discuss how the hypotheses of drug

resistance related to each other, we can first attempt to categorize them into three groups of hypotheses. The first of these categories, the *bottom-up hypotheses*, includes the gene variant, the epigenetic, and the neuroinflammation hypotheses. These hypotheses offer a primary cause for the emergence of drug resistance, which may serve as the molecular mechanism or underlying cause for the alterations found in the other hypotheses. The secondary or *drug-level hypotheses* include the target, transporter, and pharmacokinetic hypotheses, which present drug-interaction level alterations found in drug-resistant patients as the direct cause of their pharmacoresistant status. The last category, the *top-down hypotheses*, includes the intrinsic severity and neural network hypotheses, which deal with epilepsy-related alterations as the cause and consequence of drug resistance and the lack of seizure control.

The categories proposed here are based on the hierarchical and complex way in which the central nervous system (CNS) is organized: from genes and regulatory networks; to proteins, protein-to-protein interactions, and signaling pathways; to cells, neurons, synapses, and neuronal circuits; and finally, to the brain and whole-brain dynamics, behaviors, and dysfunction (Fig. 4.1) (Mahoney et al. 2019; Scott et al. 2018; Servilha-Menezes and Garcia-Cairasco 2022; Tejada et al. 2013).

Next, we will briefly present the core ideas behind each of the most studied hypotheses before discussing how the current evidence links them together.

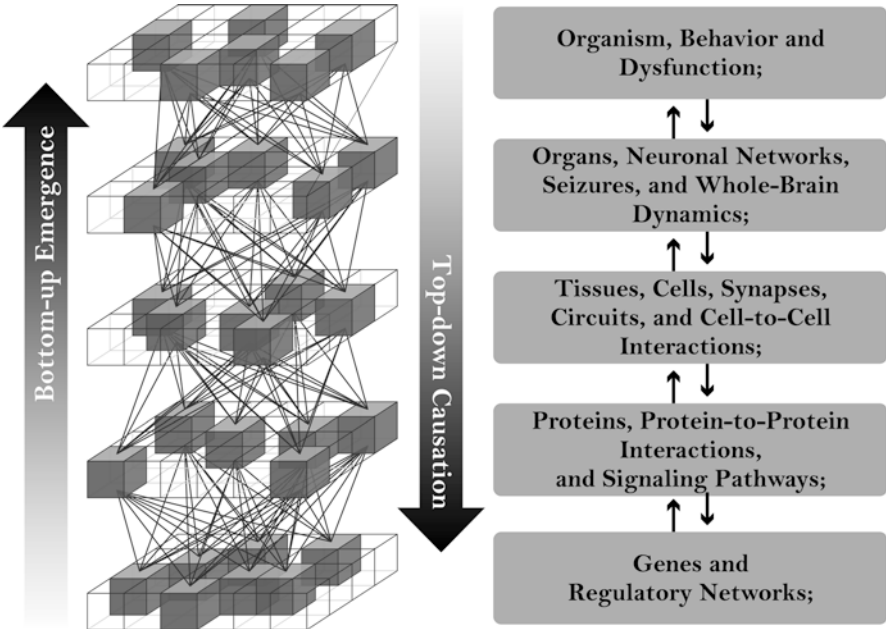


Fig. 4.1 The multiscale “network within networks” organization of the brain in epilepsy

Bottom-up hypotheses:

- *Gene Variant Hypothesis:* This hypothesis postulates that inherent resistance may result from genetic variations in proteins involved in the pharmacokinetics or pharmacodynamics of ASMs (Löscher and Schmidt 2011).
- *Epigenetic Hypothesis:* This hypothesis suggests that epigenetic changes regulating gene expression can contribute to drug resistance (Löscher et al. 2020; Pérez-Pérez et al. 2021).
- *Neuroinflammation Hypothesis:* This hypothesis states that neuroinflammation can induce blood-brain barrier (BBB) and astrocytic dysfunction, increase oxidative stress, and increase P-glycoprotein (P-gp) expression (Bazhanova et al. 2021; Löscher et al. 2020).

Drug-level hypotheses:

- *Target Hypothesis:* This hypothesis proposes that functional and structural alterations in the molecular targets of ASMs alter their sensitivity to drugs, thus leading to pharmacoresistance (Remy et al. 2003; Remy and Beck 2006).
- *Transporter Hypothesis:* This hypothesis postulates that drug resistance originates from an increase in the expression of efflux transporters at the BBB level, resulting in fewer ASM molecules available to their targets (Kwan and Brodie 2005; Löscher and Potschka 2005). The pioneering reference to this hypothesis was from Tishler et al. (1995), followed by Sisodiya et al. (1999, 2002).
- *Pharmacokinetic Hypothesis:* This hypothesis suggests that peripheral overexpression of efflux transporters and/or drug-metabolizing enzymes increases the clearance of ASMs, reducing bioavailability and therefore resulting in drug resistance (Lazarowski et al. 2007). The pioneer references showing high brain expression of P-gp related to persistently low levels of ASM were Lazarowski et al. (1999, 2004).

Top-down hypotheses:

- *Neural Network Hypothesis:* This hypothesis refers to seizure-induced alterations in the neural network, such as neurodegeneration, axonal sprouting, synaptic reorganization, aberrant neurogenesis, and gliosis, which impair the endogenous antiepileptic system and restrict ASMs from accessing neuronal targets (Remy et al. 2003; Remy and Beck 2006).
- *Intrinsic Severity Hypothesis:* This hypothesis suggests that epilepsy-related neurobiological factors associated with seizure severity are also determinants of drug responsiveness (Rogawski 2008, 2013).

The *bottom-up hypotheses* provide mechanisms of drug resistance that are based on genes, regulatory networks, protein-to-protein interactions, and signaling pathways, which may serve as the basis for the higher-level alterations observed in other hypotheses. By definition, the gene variant hypothesis is associated with changes that may affect the pharmacokinetics of ASMs. Polymorphisms in the efflux transporters ABCB1 (P-gp) and ABCC2 (multidrug resistance protein 2) genes have been demonstrated to be associated with drug resistance (Chouchi et al. 2017). Additionally, the ABCB1 gene polymorphism C3435T, associated with the

downregulation and decreased activity of P-gp in the intestines, and polymorphic CYP2C9 have been demonstrated to affect phenytoin blood concentrations (Kerb et al. 2001). These results show an association between the gene variant and the pharmacokinetic and transporter hypotheses. Single nucleotide polymorphisms of the chemokine C-C Motif Ligand 2, a chemoattractant of monocytes and macrophages, are associated with DRE in Chinese children (Campos-Bedolla et al. 2022; He et al. 2013). In another study in the Chinese population, a polymorphism (G82S) in the receptor for advanced glycation endproducts (RAGE), an important inflammation-related receptor found to be upregulated in epilepsy, was identified as a factor associated with an increased risk of DRE, thereby connecting the neuroinflammation and gene variant hypotheses (Guo et al. 2016). Changes in the inflammatory pathway, such as epilepsy-related changes in interleukin-1 β and cyclooxygenase-2, have also been linked to P-gp upregulation, which is one of the primary pieces of evidence supporting the neuroinflammation hypothesis (Bazhanova et al. 2021; Löscher et al. 2020). The first reference showing that glutamate activates COX2, inducing P-gp overexpression, was from Bauer et al. (2008).

Epigenetics is one of the major regulators of gene expression and another compelling mechanism probably associated with the drug-resistant phenotype (Hauser et al. 2018; Löscher et al. 2020; Pérez-Pérez et al. 2021). In fact, we speculate that the epigenetic hypothesis is likely linked to a majority, if not all, of the mechanisms proposed in other hypotheses. Epigenetic changes in a variety of genes, ranging from neurotrophic factors, such as BDNF, to neurotransmitter receptors, such as GluR2, have been described in animal models of epilepsy and samples from PWE, including brain samples resected from pharmacoresistant patients (Hauser et al. 2018). Furthermore, many ASMs, such as carbamazepine, oxcarbazepine, lamotrigine, phenobarbital, valproic acid, vigabatrin, gabapentin, lacosamide, levetiracetam (LEV), and cannabidiol, have been found to induce epigenetic alterations (Fonseca-Barriendos et al. 2022; Navarrete-Modesto et al. 2019), although it is important to note that the functional consequences of most of these alterations are largely unknown. Interestingly, a sizeable number of genetic variants of genes involved in DNA methylation (e.g., MECP2, MBD5, and NEUROD2), histone modification (e.g., EHMT1 and KANSL1), and chromatin remodeling (e.g., ATRX and CHD2) are risk factors or genetic determinants for epilepsy, with some being associated with drug resistance (van Loo et al. 2022). These data point to epigenetics as an important link between different hypotheses. Nonetheless, further research is required to elucidate how these changes may contribute to the development of pharmacoresistance.

The secondary hypotheses present mechanisms that work at the drug level, affecting pharmacodynamics (i.e., drug targets, transducers, and downstream effectors) and pharmacokinetics (i.e., transporters and metabolizing enzymes). In contrast, top-down hypotheses propose that DRE results from higher-level epilepsy-related alterations such as seizures and network abnormalities. Because the hypotheses in both categories work at higher levels of integration, they rely on

cellular and molecular processes, which are probably linked to the hypotheses already discussed.

The target hypothesis deals mainly with the pharmacodynamic aspect, in which loss of therapeutic efficacy is a result of changes in the structure and/or function of the targets of ASMs (Remy et al. 2003; Remy and Beck 2006). Linking the target and intrinsic severity hypotheses, neurotransmitter receptors and ion channels, common targets of ASMs, may have their activity or expression affected by seizures. Voltage-gated sodium channels (VGSC) are targets of ASMs such as phenytoin, carbamazepine, and lamotrigine. In vitro studies have demonstrated that the use-dependent block of VGSC, a mechanism of action of carbamazepine, is lost in some drug-resistant patients and the SRS stage of the pilocarpine rat model, increasing sodium current in hippocampal neurons (Remy et al. 2003). Another study on the pilocarpine model of epilepsy attributed this sodium current change to the reduced expression of the VGSC accessory subunits $\beta 1$ and $\beta 2$ (Ellerkmann et al. 2003). Further studies on transgenic mice homozygous for the VGSC $\beta 1$ gene (SCN1B) variant C121W demonstrated that these mice display and may model many of the clinical and pharmacosensitivity features of human Dravet syndrome (Reid et al. 2014). In line with this evidence, in a study on Egyptian children with idiopathic generalized epilepsy, the genetic variant C588T of the gamma-aminobutyric acid type A receptor gamma 2 subunit (GABARG2) was associated with epilepsy and a four-fold higher likelihood of pharmacoresistant syndrome (Abou El Ella et al. 2018). All things considered, it is essential to mention that even though changes in the VGSCs and GABA receptors may play an important role in the sensitivity to ASMs that target these ion channels, these changes do not explain drug resistance to ASMs with other mechanisms of action and therefore are not adequate to explain multidrug pharmacoresistance (Fonseca-Barriendos et al. 2022; Tang et al. 2017). Target changes could, however, be a consequence of dysregulation in various cell regulatory processes such as epigenetics or neuroinflammatory pathways, as already mentioned, as well as oxidative stress and changes in autophagy and metabolism. All of these processes are affected by epilepsy and are associated with seizures, either as a cause or consequence (Chang and Zou 2020; Martinc et al. 2012; Meng et al. 2013; Pearson-Smith and Patel 2017a, b; Puttachary et al. 2015; Schmidt and Löscher 2005). Furthermore, these processes cause widespread changes in expression and post-translational modifications and, therefore, may be linked as underlying mechanisms not only to the target hypothesis but also to the pharmacokinetic, transporter, and *top-down hypotheses* of drug resistance.

Sharing a similar rationale, both the transporter and pharmacokinetic hypotheses propose mechanisms of drug resistance that hinder the drug's ability to reach its target, with the first focusing more on the brain and the other on the peripheral organs (Kwan and Brodie 2005; Lazarowski et al. 2007; Löscher and Potschka 2005). Overexpression of multidrug efflux transporters, such as members of the ATP-binding cassette (ABC) superfamily (e.g., P-gp, multidrug resistance protein 2, and breast cancer-related protein) in the BBB and organs such as the intestines and the kidney have been described in numerous studies, both in drug-resistant patients and animal models of epilepsy, and offers robust clinical and experimental

data supporting these hypotheses (reviewed in Czornyj et al. (2022), Löscher et al. (2020), and Vázquez and Fagiolino (2022)). As previously discussed, genetic variation and inflammation may affect the expression and function of ABC transporters, linking the transporter and pharmacokinetic hypotheses to the gene variant and neuroinflammatory hypotheses.

Ascending on the complexity scale, clinical evidence in patients with temporal lobe epilepsy (TLE) is one of the major supporters of the neural network hypothesis. Hippocampal mossy fiber sprouting and hippocampal sclerosis are common findings in TLE frequently associated with DRE (Fang et al. 2011; Löscher et al. 2020; Schmidt and Löscher 2005). Additionally, genes involved in the cytoskeleton, synaptic plasticity, and structural/cellular reorganization, including many genes associated with the growth cone, are abnormally expressed in DRE patients compared to control patients without epilepsy. Nonetheless, the lack of a control group with pharmacoresponsive epilepsy is a significant limitation of these studies since these could be epilepsy-related changes and, therefore, not specific to pharmacoresistance (Li et al. 2009; Xi et al. 2009). Regarding these changes, neuroinflammation, epigenetic changes, and other regulatory processes can affect gene expression and may result in neural network alterations. However, evidence supporting a connection between these mechanisms and pharmacoresistant-specific changes is lacking.

Finally, the intrinsic severity hypothesis proposes that pharmacoresistance and epilepsy are based on the same neurobiological factors, in which DRE is a more severe form of the disease (Rogawski 2008, 2013). This hypothesis is mainly supported by clinical findings, in which a high frequency of seizures before treatment is a major predictor of pharmacoresistance. Other factors include a family history of epilepsy and the etiology of epilepsy. However, it is essential to note that these factors are not definite determinants, as there are cases in which patients with frequent seizures have adequate control, whereas drug-resistant patients may have had a low frequency before ASM treatment (Rogawski 2013). A similar result was observed during the SRS phase in the basolateral amygdala stimulation rat model. In this study, the researchers observed higher seizure frequency in non-responder animals than in responders following treatment with phenobarbital, where again, not all non-responders presented higher seizure frequency (Löscher and Brandt 2010). Linking the intrinsic severity and neural network hypotheses, a study by the same research group found hippocampal neurodegeneration to be an important finding observed in non-responders but not in pharmacoresponsive animals (Volk et al. 2006). Furthermore, linking the intrinsic severity to the pharmacodynamic and transporter hypotheses, seizure-induced hemodynamic changes and shear stress at the level of the BBB may affect the expression of CYP enzymes (CYP3A4, CYP2C9, CYP2C19, CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2E1, CYP2J2) and multidrug transporters (P-gp, MRP5, MRP1) (Ghosh et al. 2010, 2011).

In summary, we have discussed many theoretical ways in which different hypotheses might be associated. For each of these hypotheses, there are necessary considerations in terms of scientific plausibility (Löscher et al. 2020; Tang et al. 2017), but perhaps what is most important are not the hypotheses themselves but the

underlying mechanisms and supporting evidence on which they rely. The complex integration between the different mechanisms and multiple alterations found in pharmacoresistant patients may hide the solution to drug resistance. Therefore, the following topics will discuss how complex system-based approaches may help solve this puzzling problem.

4.3 The Role of Comorbidities in DRE

Beyond the challenge of understanding the underlying mechanisms of DRE, many additional factors of clinical relevance must be considered in the diagnosis and treatment of epilepsy and pharmacoresistance. In basic sciences, these clinical factors need to be translated into animal models used for drug screening and the modeling of epilepsy. Neurological, neuropsychiatric, and somatic comorbidities in PWE are among the factors that deserve attention, as evidence suggests that they are linked to DRE (Hitiris et al. 2007; Jansen et al. 2019; Keezer et al. 2015). In this sense, over the last few decades, commendable steps have been taken by the International League Against Epilepsy (ILAE) to include neurobiological, cognitive, psychological, and social consequences as an integral part of the definition (Fisher et al. 2005, 2014).

Epidemiological studies indicate that some conditions are significantly more prevalent in PWE than in the overall population. This is especially true for psychiatric disorders, such as anxiety, depression, bipolar disorder, attention deficit hyperactivity disorder, sleep disorders, and movement disorders; pain disorders, such as migraine, chronic pain, fibromyalgia, and neuropathic pain, and other neurological disorders, such as Alzheimer's and Parkinson's diseases and vascular dementias, as well as somatic and infectious diseases (Gaitatzis et al. 2012; Keezer et al. 2016; Ottman et al. 2011). Adding further complexity to this problem, epilepsy and comorbidities can be associated in numerous ways; for example, they may have a shared risk factor, such as in the case of many genetic syndromes, or have a causal association, in which a condition may cause or contribute to producing the other condition. This causal relationship between conditions can be direct when disease-related alterations have straight influences on the other (e.g., uncontrolled recurrent seizures can increase the risk for aspiration pneumonia and seizure-related injuries) or indirect, such as in iatrogenic comorbidities in which adverse effects of a condition's therapy contribute to or lead to the development of another (Brooks-Kayal et al. 1998; Keezer et al. 2016).

However, these different modes of association between epilepsy and comorbidities suggest no elucidation of how some comorbidities could lead to DRE. Despite this, evidence supports an association between psychiatric comorbidities and pharmacoresistance in PWE. In a study by Hitiris et al. (2007) in West Scotland, univariate and multivariate analyses of data from 780 patients (462 drug-responsive and 318 drug-resistant) with newly diagnosed epilepsy identified psychiatric comorbidities as a major predictor of pharmacoresistant status (Hitiris et al. 2007). In an

observational study on data from the Calgary Comprehensive Epilepsy Program database (data from 2573 patients), investigating the effect of depression on the risk of epilepsy and seizure outcomes has determined that PWE and depression had a significantly higher odds ratio (1.41 [95% CI, 1.03–1.96]) of failing to achieve 1-year seizure freedom. In contrast, PWE under depression therapy (antidepressants and/or counseling; an indicator of higher severity) had an odds ratio of 1.75 (95% CI, 1.06–2.94) (Josephson et al. 2017). In a smaller, cross-sectional study (Nogueira et al., 2017) stratifying for patients with mesial temporal lobe epilepsy (data from 144 PWE, of whom 82 were non-drug-responsive and 62 were drug-responsive), 68% of pharmacoresistant patients suffered from symptoms of depression and/or anxiety, an odds ratio of 2.8 (95% CI, 1.41–5.53) compared to the pharmacoresponsive group, while patients with concurrent depression and anxiety disorders presented an odds ratio of 4.04 (95% CI, 1.57–10.42). Migraine is another comorbid condition in PWE that could be implicated in pharmacoresistance. A prospective 5–10-year follow-up study in 59 patients with epilepsy and migraine and 56 with epilepsy without migraine found a negative effect of the concurrent conditions on the prognosis of epilepsy, with patients of the first group presenting a significantly longer duration of epilepsy, a lower early treatment response, and a higher incidence of intractable epilepsy and achieving remission with polytherapy (Velioglu et al. 2005).

The higher prevalence of these comorbidities in PWE and their association with pharmacoresistance may reflect shared mechanisms underlying both diseases. In the example of mood disorders, a bidirectional relationship between epilepsy and depression has been previously attributed to hyperactivity in the hypothalamic–pituitary–adrenal (HPA) axis, abnormalities in neuroanatomical structures (e.g., limbic structures such as the hippocampus and amygdala), and disturbances in neurotransmitter systems, such as serotonin, norepinephrine, glutamate, and GABA (Kanner 2008, 2012; Kanner et al. 2018).

Another critical aspect of comorbidities in DRE is related to drug choice. In essence, DRE is defined as the “failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom” (Kwan et al. 2010). An essential but somewhat overlooked aspect of this definition is that the ASMs need to be well tolerated. ASMs often produce iatrogenic symptoms that range from depression, anxiety, and behavioral/cognitive disturbances (most common) to psychosis. Conversely, some ASMs also produce psychiatric and analgesic benefits (Kanner 2016). Additionally, medications commonly used to treat psychiatric conditions, such as selective serotonin reuptake inhibitors and serotonin-norepinephrine reuptake inhibitors, help to reduce seizure frequency in PWE affected by these comorbidities, while other drugs, such as clozapine, olanzapine, clomipramine, alprazolam, and bupropion, are associated with increases in seizure frequency (Alper et al. 2007; Kanner 2016). The association between comorbidities and DRE is a subject that still requires much investigation. New studies should focus on elucidating the mechanisms underlying the epidemiological data discussed here and

how comorbidity-related pathological alterations might interact with the mechanisms of pharmacoresistance discussed above.

4.4 Systems Biology: Dealing with the Multiple Mechanisms of DRE

Systems biology represents a rather innovative, although not exactly new, approach to biology. Rather than focusing on specific elements, it “investigates the behavior and relationships of all of the elements in a particular biological system while it is functioning.” In this sense, biology is organized and operates at multiple hierarchical levels (Ideker et al. 2001). While each of these levels is complex by itself, as complexity may be understood as having a structure with variations (Garcia-Cairasco 2009), the complexity of the whole is defined by the network of interactions between all levels. In this sense, “in the particular case of the brain, we are dealing with a system with a large number of interacting components with nonlinear dynamics, self-organized to give emergence to different scales of complexity” (Servilha-Menezes and Garcia-Cairasco 2022). Although this structure may be very robust, in the sense that many alterations produce little effect on the system, it is also very susceptible to minimal perturbations in critical nodes (Ideker et al. 2001; Servilha-Menezes and Garcia-Cairasco 2022).

As aforementioned, epilepsy is a complex disease, but it is complex for far more reasons than just its multifactorial nature. Epilepsy involves dysfunctions on all levels of the organization, from genes to the behavioral-level manifestation of seizures and the many accompanying neuropsychological and somatic comorbidities (Margineanu 2012). This complexity helps to explain why, although dozens of new ASMs have been produced over the last decades, introducing many improvements in terms of ASMs’ tolerability and safety, rates of DRE have remained essentially unchanged over the years (Kalilani et al. 2018). In this sense, systems biology is an approach that may help understand epilepsy pathophysiology in its entirety and may help compose the diverse mechanisms of pharmacoresistance into a more cohesive, although complex, theory. However, a paradigm-breaking approach must be taken in epileptology to achieve this (Garcia-Cairasco et al. 2021). The revolution of high-throughput omics has changed biology since its inception. However, at the same time, the massive amount of data produced by these methods presents both an opportunity and an analytical problem: How to integrate thousands of genes variants, RNA and protein expression levels, and millions of epigenetic markers? One solution to this problem is using complex network theory to integrate the large dimensionality of independent variables in multi-omics data. One of these approaches, called *weighted gene coexpression network analysis*, is based on the idea that biochemical networks are not truly independent since the different components interact with each other. The networks of interactions are then converted into clusters, in which highly connected molecules form functional modules,

allowing for statistical analysis of whole modules instead of individual molecules. A significant advantage of this approach is that it is essentially data-driven (non-hypothesis-driven) and requires no previous knowledge about biological function and, therefore, may help understand novel biomolecular mechanisms associated with epilepsy's pathophysiology and pharmacoresistance (Bruxel et al. 2022; Mahoney et al. 2019). Other methods being used in epilepsy research include machine and deep learning techniques, principal component analysis, multidimensional scaling, and hierarchical cluster analysis. Nevertheless, despite these advancements, it is important to point out that many of those methodologies and techniques are still in development and will therefore require substantial investment in infrastructure, development, validation, and, obviously, the qualification of individuals to work with these new resources.

A core limiting factor for advancing systems biology and computational approaches in epilepsy research (but also in other fields) is the lack of data availability. Big data and *non-cryptic* data sharing have the potential to change the way we do research in epilepsy, although numerous privacy, legal, and ethical issues still need to be considered in order to make such projects come to fruition (Lhatoo et al. 2020). In this sense, another subject that requires attention and resources is the creation of databases for neuroscience and epilepsy in the form of large data repositories for different types of data, both from patients and animal models, going beyond just molecular data (e.g., genomic, epigenomic, transcriptomic, proteomic, and metabolomic data; omics in general) to also include neuronal activity, connectivity and structure, imaging, behavior, and many other types of data and metadata about the subject (e.g., age, weight, experimental design, and, in the case of patients, medical records). However, for these data to achieve their maximum usefulness, they must be integrated under unique identifiers for each human or animal subject, allowing data to be “findable, accessible, interoperable, and reusable” (FAIR principles) (Wilkinson et al. 2016). Moreover, they also need to be organized coherently in a common framework that reflects the hierarchical *network within networks* organization of the brain and epilepsy/comorbidities. In this regard, the Kavli Foundation started the *Neurodata Without Borders* (NWB) consortium, an initiative promoting data standardization in neuroscience, to facilitate data sharing among neuroscientists (<http://nwb.org>) (Rübel et al. 2022). This initiative aims to make data easily “shared, pooled, and analyzed,” allowing neuroscientists to take full advantage of the massive amount of data produced by large-scale brain research projects such as the U.S. BRAIN Initiative (<https://braininitiative.nih.gov/>; Insel et al. 2013) and the Allen Institute for Brain Science (<https://alleninstitute.org/>). Advancements in the NWB project have been published recently by Rübel et al. (2022), who reported the development of data management, analysis, visualization, and archive tools built for the NWB data language. Although NWB is centered around neurophysiological recordings, NWB files may contain all the multimodal measurements collected in a single experiment (e.g., simultaneous recording of neurophysiological, behavioral, and stimulation information) while also keeping pertinent metadata about the subject, experimental design, and acquisition settings. NWB is also flexible in the sense that it enables the creation of user-defined

extensions that allow for new and specialized data types and the incorporation of new use cases. Additionally, a central aspect of NWB software is that it is *open-source*, meaning that it is freely available for use or modification (Rübel et al. 2019, 2022). Finally, and perhaps most importantly, Rübel et al. (2022) also reported the development of the *Distributed Archives for Neurophysiology Data Integration* (DANDI), a web-based data archive (<https://dandiarchive.org>) designed to store cellular neurophysiology data with the NWB as its core data language. DANDI is also compatible with other data standards such as *Brain Imaging Data Structure* (BIDS; <https://bids.neuroimaging.io/>) and *Neuroimaging Data Model* (NIDM; <http://nidm.nidash.org/>).

Other examples of neuroscience-centered databases include the *Neuroscience Multi-Omic Archive* (NeMO Archive; <https://nemoarchive.org/>), a data repository focused on *omic* data generated from the BRAIN Initiative and related brain research projects (Ament et al. 2022); the *Brain Observatory Storage Service and Database* (BossDB; <https://bossdb.org/>) is focused on the storage, accesses, and processing of multidimensional and volumetric neuroscience datasets from Electron Microscopy (EM) and X-Ray Microtomography (XRM) and other imaging techniques (Hider et al. 2022); and the *Brain Image Library* (BIL; <https://www.brainimagelibrary.org/>), which focus on the storage of high-resolution, high-quality brain microscopy datasets created by a variety of technologies such as two-photon tomography (STPT), fluorescence micro-optical sectioning tomography (fMOST), oblique light-sheet tomography (OLST) (Benninger et al. 2020).

Regarding epilepsy and pharmacoresistance, a couple of crucial considerations need to be discussed about the new resources presented here. First, the recent development of these new data repositories, file formats, software, and associated infrastructure is the direct consequence of public policies and funding dedicated to understanding the brain. However, it also reflects the maturity of technological and research advances made in the last few years in *omics*, microscopy, electrophysiology, and other techniques, as well as in terms of computational power for processing and storing these massive datasets (Insel et al. 2013). Additionally, computational neuroscience and deep/machine learning techniques have dramatically advanced in the last decades and have the potential to revolutionize neuroscience, epilepsy research, and health care. Nevertheless, these techniques rely on the availability of large and high-quality datasets to achieve optimal results, further highlighting the need for these data repositories (Rasheed et al. 2021; Richards et al. 2019). Second, most of these resources are *open-source* and may, therefore, be used as is for epilepsy research but, ideally, may also be used to create platforms focused on epileptology for storing, accessing, and processing molecular, pharmacology, electrophysiology, imaging, behavior, and other types of data, allowing for greater collaboration among scientists from different countries and institutions.

In summary, empowered by new computational resources, systems biology emerges as a powerful approach to epilepsy, allowing us to harness knowledge from complexity and, therefore, help in the search for the *complex solution* required to the *complex problem* of multidrug pharmacoresistance.

4.5 The Advent of Systems Pharmacology for the Treatment of Epilepsy

In contrast to the precedent, reductionist view of a target-focused drug discovery paradigm (synthesized under the “one gene, one drug, one disease” motto), systems (or network) pharmacology perceives complex, multifactorial disorders as robust states; under that assumption, systems-level stability may well be the cause of some drug-resistant phenotypes (Csermely et al. 2013; Hopkins 2008). Therefore, they are unlikely to be controlled by single-point interventions. In fact, it has been observed that many long-used treatments for complex neurological and psychiatric disorders are unintended multi-target drugs that emerged from *target-agnostic drug discovery* studies during the phenotypic screening era (Bianchi et al. 2009; Margineanu 2016). There are many ways in which systems pharmacology can be exploited to deliver new and improved therapies or to make better use of existing ones, from a more rational choice of drug targets to novel paradigms of drug design (Csermely et al. 2013). However, this may imply shaking some installed ideas inherited from the target-focused drug discovery paradigm, among them the notion that the strongest single-target intervention is the best (or, in other words, that exquisitely selective drug candidates as potent as possible should be pursued) (Talevi 2016).

The most salient way to integrate the network pharmacology perspective into drug discovery and development is possibly through the use of appropriately chosen drug combinations or the design of *tailored multi-target drugs*. This is not only of interest as a strategy to address the intrinsic complexity of the disease but also sounds like a natural answer to the target hypothesis of pharmacoresistance, considering that it is unlikely that two distinct drug targets will simultaneously lose sensitivity to drugs. Moreover, the restoration of a healthy state from a systems-level stable disease state may be better achieved by simultaneous weak/partial attacks on multiple proteins (Bianchi et al. 2009; Csermely et al. 2013). For example, partial agonists have recently attracted much interest for the treatment of several complex neuropsychiatric conditions such as schizophrenia, depression, anxiety, and addiction (Peris and Szerman 2021). Low-affinity multi-target ligands such as memantine or LEV are also useful to exemplify this point (Contreras-García et al. 2022; Klitgaard et al. 2016; Zheng et al. 2014): *stronger is not always better*. The use of partial agonists and low-affinity ligands may be advantageous in terms of enhanced efficacy and safety. This is a major point to consider in view of the high proportion of failed drug development projects due to an unacceptable safety profile (Cook et al. 2014). In the case of epilepsy, it should be noted that the diagnosis of DRE involves the failure of *two well-tolerated* pharmacological interventions (Kwan et al. 2010), which means that efficacious interventions might be disregarded owing to intolerable side effects.

With some remarkable exceptions, including recently approved and investigational ASMs, the multi-target approach has scarcely been implemented in the field

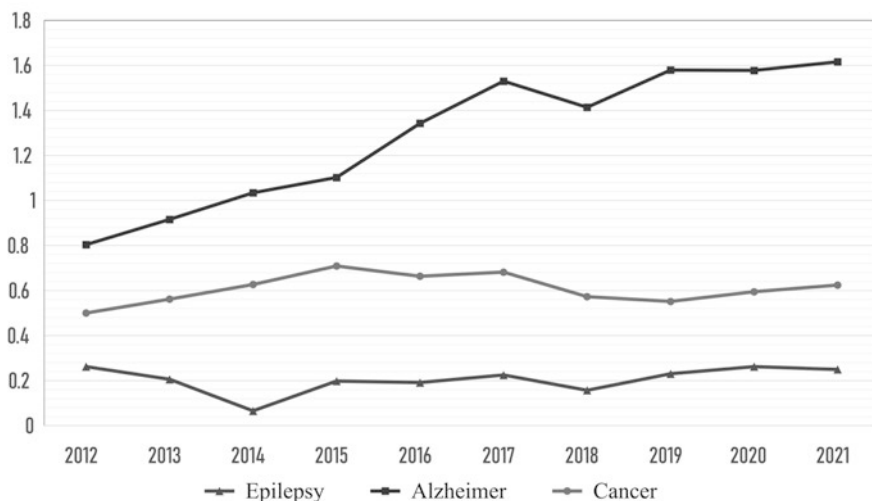


Fig. 4.2 The number of articles containing the words “epilepsy”/“cancer” / “Alzheimer” AND “hybrid drug” OR “hybrid compound” OR “multi-target” OR “multi-target” in the title, keywords, or abstract (source: Scopus); the data have been weighted by the total number of articles containing the words “epilepsy” / “cancer” / “Alzheimer” and expressed as a percentage. It can be appreciated that the proportion of articles embracing the network pharmacology perspective in the epilepsy field is surprisingly low compared to other complex conditions

of epilepsy in comparison to other complex disorders (Fig. 4.2), although this scenario seems to be readily changing in recent years.

Any discussion on the potential benefits of systems pharmacology in epilepsy should possibly begin with LEV, which is a second-generation, first-in-class ASM that is structurally unrelated to previously approved ASMs and also possesses a distinctive mechanism of action (Contreras-García et al. 2022; Crepeau and Treiman 2010). LEV was approved by the United States Food and Drug Administration in 1999 as an add-on therapy for focal-onset seizures in adults (and subsequently in children), myoclonic seizures in adults and adolescents older than 12 years with juvenile myoclonic epilepsy, and generalized-onset seizures. The European Medical Association approved its use for the treatment of focal-onset seizures and focal to bilateral tonic-clonic seizures (as monotherapy) and as adjunctive therapy for focal-onset seizures, myoclonic seizures, and generalized-onset tonic-clonic seizures (Contreras-García et al. 2022). Currently, it is considered a broad-spectrum ASM, used either alone or in combination therapy (Hakami 2021). Its *off-label* use as second-line therapy for *status epilepticus* has also been explored in randomized controlled trials, exhibiting similar efficacy to fosphenytoin or phenytoin in cessation rate and drug resistance and seemingly superior performance in terms of pooled safety (Feng et al. 2021). Other *off-label* uses include neuropathic pain, movement disorders, and headaches (Crepeau and Treiman 2010). LEV is a repurposed drug originally (and unsuccessfully) investigated as a nootropic agent and later identified as an ASM using an audiogenic mouse model of seizures

(Rogawski 2008). LEV displays protective activity in a variety of animal models of seizure and epilepsy (Contreras-García et al. 2022) but, of most interest, failed to elicit protective effects in the two most traditional and widely used preclinical models of seizure, the Maximal Electroshock Seizure and the Pentylenetetrazol tests. This implies, as a corollary, that limiting the options of animal models used as primary in vivo screening might not only disregard valuable drug candidates but also lead to new drugs with redundant pharmacological profiles (D'Ambrosio and Miller 2010; Löscher 2011). Many pharmacological targets besides SV2A have been described for LEV (Contreras-García et al. 2022), including Alpha-Amino-3-Hidroxy-5-Methyl-4-Isoxazole Propionic Acid (AMPA), adenosine, noradrenaline, and serotonin receptors, targets involved in calcium homeostasis, and others. As previously mentioned, LEV acts on them as a low-affinity ligand.

More recently approved multi-target compounds include cannabidiol (CBD) and cenobamate. CBD has recently been approved as an add-on therapy for intractable childhood-onset seizures, including those associated with Dravet syndrome, Lennox-Gastaut syndrome, and tuberous sclerosis complex (Britch et al. 2021). Remarkably, it has a very complex pharmacology, with over 60 pharmacological targets described in the literature, among them several ion channels (e.g., TRP channels) and receptors (e.g., glycine and serotonin receptors) with a possible role as targets for ASMs (Ibeas Bih et al. 2015). In contrast, cenobamate has a narrower pharmacological scope: it is a tailored dual agent that blocks voltage-operated sodium channels and acts as a positive allosteric modulator of the GABA_A receptor. Interestingly, a post hoc analysis of a subset of patients from a long-term multicenter phase 3 open-label study showed high rates of sustained 100% and $\geq 90\%$ seizure reduction, and almost half of the patients who chose to continue on adjunctive cenobamate after the study ended achieved seizure freedom for at least 12 months (Sperling et al. 2021). The patients enrolled in this phase 3 study had been diagnosed with focal epilepsy and had previously failed to achieve seizure freedom despite being treated with stable doses of up to 3 ASMs.

A renewed interest in rational polytherapy for drug-resistant epilepsy has also been perceived in recent years. Previously, monotherapy was preferred over polytherapy under the belief that drug combinations did not provide substantial benefits in terms of efficacy but posed higher risks in terms of potential drug-drug interactions. This made sense for old ASMs, which are known enzyme inducers (e.g., phenytoin and carbamazepine) and/or inhibitors (e.g., valproic acid), but is not necessarily valid when considering the growing number of new ASMs, characterized by improved tolerability, reduced participation in drug-drug interactions, and a wider therapeutic window (Lee et al. 2019). Moreover, whereas classical ASMs are limited regarding their main mechanism(s), newer drugs offer innovative modes of action and are less likely to overlap mechanistically when used in combinations. For instance, fenfluramine was recently approved as an add-on therapy to treat seizures in severe epileptic syndromes, Dravet and Lennox-Gastaut, and its pharmacodynamics are unlike any other approved ASD. It is believed that it exerts its antiseizure effects through the serotonergic system by disrupting the vesicle storage of serotonin and by inhibiting its reuptake; moreover, its major component,

norfenfluramine, shows agonism with different subtypes of serotonin receptors (Balagura et al. 2020; Sourbron et al. 2017). An additional mechanism involving $\sigma 1$ -receptors also seems to be at play, potentiating the antiseizure activity of this drug (Park et al. 2019; Rodríguez-Muñoz et al. 2018). Importantly, new ASMs typically undergo randomized controlled clinical trials for add-on therapy in patients with drug-resistant epilepsy. Accordingly, they are primarily used in combination therapies and are only occasionally compared to conventional ASMs to be approved as monotherapies for newly diagnosed epilepsy (Park et al. 2019).

Combination therapy should achieve supra-additive effects (synergism) regarding the efficacy and only additive effects with respect to adverse events, or alternatively, additive effects regarding the efficacy and infra-additive effects in relation to adverse events (Lee et al. 2019; Park et al. 2019). This is more likely to be achieved with combinations of drugs with different mechanisms of action and no pharmacokinetic drug-drug interaction. Combinations of drugs having the same or similar mechanisms (e.g., a combination of sodium channels blocking agents) tend to be associated with a higher incidence of adverse events and treatment discontinuation (Barcs et al. 2000; Margolis et al. 2014; Sake et al. 2010). On the contrary, the combination of valproic acid with lamotrigine has consistently shown superior efficacy compared with different monotherapies (see, for instance, Brodie and Yuen 1997; Lee et al. 2018; Pisani et al. 1999), and some common combinations supported by case series or observational studies include valproate and ethosuximide, lacosamide and LEV, and lamotrigine and LEV (Park et al. 2019), all pairs being characterized by the distinctive mechanisms of the combined drugs.

Very recently, Löscher and coworkers reported a series of preclinical studies that explicitly consider the network-pharmacology paradigm to select and test combinations of ASMs (Klee et al. 2015; Schidlitzki et al. 2020; Welzel et al. 2019, 2021). For instance, the topiramate/LEV combination was chosen using network analysis, and its efficacy was assessed in the kainate model of acquired temporal lobe epilepsy by administering both drugs in the latency period of the model before the establishment of SRS (Schidlitzki et al. 2020). It was shown that the rationally chosen combination reduced seizure load based on the severity, duration, and frequency of electroclinical seizures. The combination proved more efficacious than either of the combined drugs alone, and it was also superior to a randomly chosen combination of drugs (LEV plus phenobarbital). The most recent report from this group is even more surprising: eight novel rationally chosen combinations of 14 drugs with mechanisms that target different epileptogenic processes were tested by administering them again during the latent epileptogenic period in the previously mentioned kainate mouse model (Welzel et al. 2021). Compared to vehicle, the most effective drug combination consisted of LEV, atorvastatin (a cholesterol-lowering drug), and ceftriaxone (an antibiotic), which markedly reduced the incidence of electrographic seizures and electroclinical seizures, demonstrating the power of systems pharmacology to identify non-obvious, unforeseen drug combinations with antiepileptogenic potential.

It should be noted that the rational combination of drugs should not only consider the pharmacodynamic aspects of the combined treatments but also their reciprocal influences on pharmacokinetics. As previously mentioned, older ASMs are

more likely to participate in clinically relevant drug-drug interactions of a pharmacokinetic nature. Recently, for example, it was reported that valproic acid can induce ABCG2/rBCRP overexpression in BBB endothelial cells via activation of the PPAR γ receptor (Kukal et al. 2022), which could harm the central nervous system drug bioavailability of any substrate for ABCG2 given concomitantly.

4.6 Conclusion

Before concluding our discussion, we would like to add that the complexity of DREs is not only inherent to the intricate mechanisms of pharmacoresistance but also a consequence of the challenges of defining and diagnosing epilepsy, seizures, and the numerous associated comorbidities, as well as their treatment (Servilha-Menezes and Garcia-Cairasco 2022). Our proposal of a complex systems approach to the mechanisms of pharmacoresistance offers an integrative view that aims to mitigate the limitations of the numerous proposed hypotheses. Finally, the computational and mathematical analysis, integration, and modeling of complex biological systems offered by systems biology is an approach that has the potential to revolutionize the way clinical and basic sciences are made, helping to advance our knowledge about how our brain works and what leads to its dysfunction. Conversely, systems pharmacology represents a paradigm shift in the direction of a network approach that, through the use of multi-target drugs, aims to modulate the entire system toward seizure control (Talevi 2022). Hopefully, these new paradigms will help to discover the long-sought solution to the puzzling problem posed by pharmacoresistant epilepsy. Many of the future advances will also be associated with the strong need for paradigm shifts, particularly in the integration of methods, neurotechnology, and big data sharing and modeling.

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Chapter 5

The Role of High-Frequency Oscillation Networks in Managing Pharmacoresistant Epilepsy



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Abstract Persons with pharmacoresistant epilepsy have a higher risk of mortality than those with controlled seizures. Seizure control can be achieved with surgical removal or electrical stimulation of the brain area responsible for generating seizures, but localizing this brain area is difficult and requires different non-invasive tests and, in some cases, an invasive EEG study. Resection or stimulation of the seizure onset zone does not always control seizures, suggesting there are other pathological areas involved in generating seizures. There is a significant body of research on high-frequency oscillations (HFO) associated with normal and abnormal brain function. In the brain with epilepsy, it is believed pathological HFO corresponds with neuronal disturbances in tissue capable of generating seizures, and their removal correlates with seizure control; thus, pathological HFO can be a biomarker of epileptogenic tissue and play an important role in the diagnosis and treatment of epilepsy. However, additional work is needed to better understand the different types of HFO, and the mechanisms generating each, and the parts of the ictal and interictal HFO networks that need to be targeted with therapy to control seizures. This chapter will discuss these issues, the current gaps in knowledge, and demonstrate how measures of HFO networks could be used in the surgical treatment of pharmacoresistant epilepsy.

Keywords Pharmacoresistant epilepsy · High-frequency oscillations · Epilepsy surgery · Seizure onset zone · Epileptogenic zone

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Abbreviations

CA1	Cornu Ammonis 1
EZ	Epileptogenic zone
FR	Fast ripple
fRonO	Fast ripple on oscillation
fRonS	Fast ripple on spike
GABA	Gamma-aminobutyric acid
HFO	High-frequency oscillation
iEEG	Intracranial EEG
IPSP	Inhibitory postsynaptic potential
LE	Local efficiency
LFP	Local field potential
MEA	Multi-electrode array
MI	Mutual information
PIN	Pathologically interconnected neurons
RDRRD	FR rate-distance network radius resection difference
RNS	Responsive neurostimulator
RonO	Ripple on “background” oscillation
RonS	Ripple on spike
RR	Resection ratio
SEEG	Stereo EEG
SOZ	Seizure onset zone
SpW-R	Sharp-wave ripple
TBI	Traumatic brain injury
ur_mLE	FR MI network unresected mean LE
γ	FR MI network characteristic path length

5.1 Introduction

Epilepsy is a serious neurological disorder associated with an increased risk of mortality, especially in people with poorly controlled seizures. About one-third of individuals diagnosed with focal epilepsy have pharmacoresistant seizures (Engel et al. 2008). Alternatives are surgical or stimulation therapy, and in either of these approaches, accurate delineation of the brain area responsible for generating seizures is critical to control or eliminate seizures (Bergey et al. 2015; Engel et al. 2012; Nair et al. 2020; Spencer et al. 2005, 2018; Téllez-Zenteno et al. 2005, 2007). EEG tests, both scalp EEG and intracranial EEG (iEEG), can help identify the seizure onset zone (SOZ) and where seizures rapidly propagate, which are important components of the epileptogenic zone (EZ). The EZ can be defined as the brain area

necessary and sufficient for generating seizures and the minimal volume of tissue that needs to be removed to eliminate seizures (Jehi 2018; Rosenow and Lüders 2001).

Currently, the EZ cannot be directly measured; rather, it is inferred from the results of non-invasive EEG tests, MEG, MRI, PET, and neuropsychological tests, and when required, invasive EEG tests (Zijlmans et al. 2019). However, removal of the SOZ alone does not always abolish seizures (Engel et al. 2012; Téllez-Zenteno et al. 2005, 2007). Similarly, positioning stimulation electrodes at or in close proximity to the SOZ is intended to be palliative and does not always control seizures (Nair et al. 2020). These results suggest there are other functional disturbances at sites near or distant from the SOZ that play a role in the generation of seizures, or possibly these sites themselves have the capacity to generate seizures. Furthermore, these results have extended the concept of the EZ, which often implies a narrow, continuous area of tissue such as a well-circumscribed lesion that generates seizures, to include an area that could be broad and discontinuous like a network (Spencer 2002). In an epileptogenic network, several distributed sites could be anatomically and functionally connected to the SOZ and play critical roles in generating seizures.

The anatomical and functional abnormalities that define the epileptogenic network are unknown, thus delineating the network is very difficult (Bartolomei et al. 2017; Englot et al. 2016; Goodfellow et al. 2016; Jirsa et al. 2017; Khambhati et al. 2016; Kini et al. 2019; Sinha et al. 2017; Sinha et al. 2020; Wilke et al. 2011). For more than two decades, researchers have studied HFO in the wide-bandwidth EEG, which consists of brief (10–200 ms) bursts of spectral energy between 80 and 600 Hz (Bragin et al. 1999a; Frauscher et al. 2017; Urrestarazu et al. 2007). HFO occurs in the normal mammalian brain (Frauscher et al. 2018a), but can be recorded in the brain with epilepsy during interictal (Bragin et al. 2002a; Bragin et al. 2002b; Bragin et al. 2003; Bragin et al. 2004; Staba et al. 2002; Urrestarazu et al. 2007) and ictal episodes (Bragin et al. 2005; Lévesque et al. 2012; Schönberger et al. 2019; Weiss et al. 2013, 2016a). Pathological HFO are strongly associated with epileptogenic tissue, and resection of them correlates with postoperative seizure outcome (Haegelen et al. 2013; Jacobs et al. 2010, 2018; Nevalainen et al. 2020). In the brain with epilepsy, pathological HFO is believed to represent neuronal disturbances responsible for seizures and could be a biomarker of the epileptogenic network (Bragin et al. 2000; Ibarz et al. 2010).

In this work, we first briefly review the different types of HFO and mechanisms generating each, discuss the role of HFO in the development of epilepsy and ictogenesis, present studies of the spatiotemporal properties of HFO with respect to the SOZ, provide evidence for an HFO network that could help define the epileptogenic network, and finally, discuss how HFO could be used in planning surgical therapies for individuals with pharmacoresistant epilepsy. Readers are encouraged to read more detailed reviews on the cellular and circuit mechanisms generating HFO (Jiruska et al. 2022) and HFOs as biomarkers of the EZ in clinical epilepsy (Frauscher et al. 2017).

5.2 Different Types of HFO in Normal Brain and the Brain with Focal Epilepsy

Fast ripples (FR) are one type of HFO and are defined by our group as bursts of spectral energy between 200 and 600 Hz that are typically 8–50 ms in duration (Weiss et al. 2018, 2021, 2022a, b, 2023). Others use a bandwidth between 250–600 Hz (Frauscher et al. 2017). Fast ripples commonly superimpose on the iEEG or local field potential (LFP) background (i.e., FR on oscillation or fRonO) or can superimpose on epileptiform spikes (Fig. 5.1a, b, i.e., FR on spike or fRonS) (Waldman et al. 2018; Weiss et al. 2018). Both types of FR are rare in brain tissue thought to be healthy (Frauscher et al. 2018b). Thus, FR is thought to indicate epileptogenic brain tissue or sometimes irritative tissue (Nevalainen et al. 2020; Weiss et al. 2022a, b, 2023). Our group (Weiss et al. 2022a, b) and others (Brázdil et al. 2017; Usui et al. 2010, 2015) found FR with power at higher spectral frequencies may be a stronger biomarker of epileptogenic tissue than FR with lower spectral frequency.

Another type of HFO is ripples that are bursts of spectral energy between 80–200 Hz and 30–200 msec in duration (Frauscher et al. 2017). Ripples also can occur on lower frequency rhythms (i.e., ripple on oscillation or RonO) or superimpose on an epileptiform spike (i.e., ripple on spike or RonS) (Waldman et al. 2018; Weiss et al. 2018). In area CA1 of the healthy hippocampus ripples (RonO) that superimpose on sharp waves in the LFP play a role in memory consolidation (Buzsáki 2015; Liu et al. 2022). Ripples that occur outside of area CA1 of the hippocampus are not associated with a sharp wave (Khodagholy et al. 2017). Additionally, high-gamma oscillations occur in animals and humans, typically during behavior, and have a frequency content (90–150 Hz) (Buzsáki and Wang 2012) that overlaps with the ripple band. A consensus has not been reached among neurophysiologists with respect to distinguishing longer-duration ripple events from

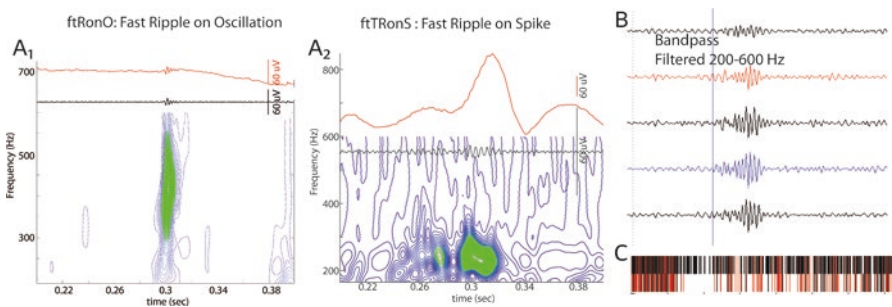


Fig. 5.1 Demonstration of the quantification of FR properties and FR temporal relationships that exhibit FR propagation. (a) Example of a FR on oscillation and FR on spike captured and quantified by our HFO detector using the topographical analysis of the wavelet convolution. (b) Example of iEEG exhibiting FR propagation from the blue trace to the red trace. (c) Raster plot of FR from these contacts demonstrating consistent propagated events (<250 ms) in red over 30 min of recording

high-gamma activity. However, high-gamma activity is thought to be of longer duration, and its amplitude waxes and wanes because it can couple with the phase of lower frequency theta wave (Canolty et al. 2006).

In experimental models of chronic epileptic seizures (Li et al., 2021a, b) and in patients with epilepsy (Jacobs et al. 2008, 2010; Urrestarazu et al. 2007), there is a high occurrence of ripples in epileptogenic and irritative tissue. However, studies found rates of ripples are a less specific biomarker of epileptogenic tissue than rates of FR (Haegelen et al. 2013; Jacobs et al. 2008, 2010), suggesting some ripples might be pathological HFO but others normal (Engel et al. 2009). Indeed, studies show ripples in epileptogenic tissue can have a higher amplitude than ripples in healthy brain regions (Guragain et al. 2018), but differences in ripple amplitude were not found in the hippocampus (Bragin et al. 2007; Weiss et al. 2020). There also appears to be a disturbance in the coupling between some ripples and delta-frequency slow/delta waves during sleep. In epileptogenic tissue, the phase of ripple coupling with slow waves occurs during the transition from UP to DOWN and from DOWN to UP transition, whereas in healthy tissue ripples have a preferred phase from the DOWN to UP transition (Ellenrieder et al. 2016; Frauscher et al. 2015; Song et al. 2017). When fRonO are generated during non-REM sleep in the SOZ, they mostly occur during the DOWN state of the slow/delta background (Weiss et al. 2022a, b, 2023). Importantly, during the DOWN to UP transition a normal hierarchy of coupling (Staresina et al. 2015) was observed between ripples and other rhythms, such as slow/delta/theta and spindles, but this was not the case during the UP to DOWN transition (Song et al. 2017).

Epileptiform spikes are not uniform in either morphology or pathological significance. Rasmussen theorized that some “red spikes” are indicative of epileptogenic tissue, while “green spikes” are indicative of irritative tissue but did not specify criteria for separating these populations (Rasmussen 1987). RonS and fRonS may represent red spikes and very red spikes, respectively. Sites generating RonS in either scalp EEG (Cai et al. 2021) or iEEG (Wang et al. 2013, 2016a, b) overlap with the SOZ, and resection of these sites correlates with better seizure control. Also, scalp RonS in children with Rolandic epilepsy predicts seizure risk better than spikes without HFO (Kramer et al. 2019). In intraoperative recording from patients with temporal lobe epilepsy, failure to resect sites generating fRonS was found to strongly predict poor seizure outcome (Weiss et al. 2018).

5.3 Mechanisms Generating Normal and Pathological HFO and the Contributions of Inhibitory and Excitatory Cells

In considering the mechanisms that generate HFOs, it is important to recognize that in hybrid macroelectrode and microelectrode recordings of the iEEG and LFP respectively, ripple and FR occur at a much higher rate and contain higher spectral frequencies in the LFP recordings than the iEEG (Schevon et al. 2009; Worrell et al.

2008). Thus, the mechanisms that generate ripple and FR recorded in the LFP and encompassing a $400\text{ }\mu\text{m}^3$ region around the electrode may involve a different mechanism than those that generate ripples and FR over several cubic millimeters of tissue, wherein there is a larger spatial summation (i.e., averaging) recorded on the macroelectrode contact of the iEEG (Buzsáki et al. 2012).

FR in the LFP can be generated by populations of pyramidal neurons or granule cells within a region thought not to extend beyond 1 mm (Bragin et al. 2002a, 2011) and synchronously firing action potentials that produce a population spike(s). Others found evidence suggesting FR occurs when two groups of synchronously firing pyramidal neurons fire out-of-phase with each other within a similarly small region (Foffani et al. 2007; Ibarz et al. 2010). However, optogenetic manipulation in *in vitro* preparations shows that FR also can be generated by synchronized chloride-mediated depolarizing postsynaptic potentials (Alfonsa et al. 2015). In simulations of the LFP (Demont-Guignard et al. 2012) and iEEG (Shamas et al. 2018), FR occurs when the chloride reversal potential is greater than the resting membrane potential. Consistent with the role of GABA-mediated depolarizing postsynaptic potential are observation of FR in epileptogenic tissue that occurs during the DOWN state (Weiss et al. 2022a, b, 2023) when inhibition may be maximal. Additional data from whole cell or LFP recordings are needed to determine if widespread FR, which are more likely to be detected in the iEEG, are associated with IPSPs (Dubanet et al. 2021).

Mechanisms of physiological ripples in the context of sharp wave-ripple complexes (SpW-R) have been mostly studied in area CA1 of the hippocampus (Buzsáki 2015). The sharp wave is only seen in the hippocampus and outside the hippocampus the mechanisms responsible for physiological ripple generation are not as well established (Khodagholy et al. 2017; Nitzan et al. 2020). During SpW-R in the murine hippocampus (Stark et al. 2014) and in the human hippocampus (Quyen et al. 2008), the firing of inhibitory interneurons and pyramidal neurons are entrained at different phases to the SpW-R in the LFP. Summated action potentials, primarily from pyramidal neurons during the SpW-R, are thought to make a substantial contribution to generating the SpW-R in the LFP (Schomburg et al. 2012). However, rhythmic IPSPs during the SpW-R also contribute to the SpW-R in the LFP (Ylinen et al. 1995). Regarding pathological ripples in the brain with epilepsy, it is unclear the differential contributions of hyperpolarizing and depolarizing postsynaptic potentials, and pyramidal cell action currents associated with ripples recorded in the LFP versus the iEEG. In hybrid depth, electrodes containing Behnke-Fried microelectrodes recordings positioned in the human epileptic hippocampus, RonO detected in the iEEG is associated with an increased firing rate of nearby excitatory neurons (Weiss et al. 2020). In paired subdural and Utah microelectrode array (MEA) recordings from the anterior temporal lobe of patients with epilepsy, the occurrence and power of RonO events in the iEEG correlates with the number and synchrony of ripples in the LFP and the number of single unit action potentials (Tong et al. 2021). While these findings are undoubtedly mechanistically important, this study did not differentiate pathologic and physiologic RonO, and uncertain

whether the action currents measured in the MEA were involved in generating the RonO in the iEEG (Schevon et al. 2009).

During epileptiform spikes, excitatory and inhibitory synaptic conductance increase, and possibly action currents also contribute to the very sharply contoured waveform in the LFP and iEEG (Huberfeld et al. 2011; Pallud et al. 2014). The polarity of the spike depends on the location of the recording electrode in relation to the spike-generating dipole, which relates to the location of cell bodies and apical and distal dendrites (Buzsáki et al. 2012). One study found the RonS and fRonS always occur during the ascending (i.e., more positive) segment of positive polarity spikes (Guth et al. 2021). However, RonS and fRonS have also been observed on negative polarity spikes, too (Weiss et al. 2016a, b, 2018). Single-unit recordings indicate more neurons (presumably excitatory cells) are active during RonS and fRonS than epileptiform discharges without HFOs (Guth et al. 2021). Macroelectrode recordings show ripple amplitude increases when ripples are tightly coupled with spikes, which might correspond with a larger recruitment of neurons (Weiss et al. 2022a, b) due to a stronger depolarizing synaptic drive. Epileptiform discharges are highly heterogeneous (Keller et al. 2010) and interictal and pre-ictal large amplitude iEEG discharges can contain HFOs despite differential recruitment of excitatory and inhibitory interneurons (Alvarado-Rojas et al. 2015; Huberfeld et al. 2011; Pallud et al. 2014; Weiss et al. 2019). More work is required to understand the mechanisms generating HFOs on spikes, which likely involves strong depolarizing excitatory drive and according to modeling studies, a role for GABA-mediated post-synaptic potentials (Demont-Guignard et al. 2012).

5.4 Fast Ripples as Biomarkers of Epileptogenic Tissue

FRs first appear in the early stages of epileptogenesis and their rate and spatial distribution can predict the development of seizures and status epilepticus in both murine chemoconvulsant models (Bragin et al. 1999b, 2000, 2002a, 2002b, 2004, 2005; Foffani et al. 2007; Ibarz et al. 2010; Lévesque et al. 2012, 2016; Sheybani et al. 2018, 2019), and in murine models of traumatic brain injury (TBI) (Kumar et al. 2021; Li et al. 2021b; Ortiz et al. 2018). One hypothesis of FR during epileptogenesis proposes that after an injury a relatively small subpopulation of pyramidal cells and inhibitory interneurons form a pathological microcircuit in tissue as small as 1 mm³ (Bragin et al. 2002a, 2011). This microcircuit of pathologically interconnected neuron (PIN) cluster can generate synchronous bursts of action potentials forming a population spike(s) detected in the LFP and iEEG (Bragin et al. 2011) as FR. This hypothesis proposes PIN clusters can act as internal kindling generators that potentiate synaptic connections in target areas and recruits additional structures (Bragin et al. 2000). Our lab (Weiss et al. 2022a, b, 2023) and others (Jahromi et al. 2021; Otárola et al. 2019) have found that FR can propagate at 1.54 mm/msec (Fig. 5.1b, c), can travel distances up to 35–40 mm (Weiss et al. 2022a, b, 2023), and propagating FR possess unique spectral frequencies and power as compared with

non-propagating FR (Weiss et al. 2022a, b, 2023). Thus, FR propagation by slow and likely poly-synaptic conduction may act as the substrate of the PIN cluster FR network and influence its evolution. PIN clusters also play an important role in ictogenesis and trigger seizures when they coalesce and synchronize (Li et al. 2019; Weiss et al. 2019). In murine chemoconvulsant models (Bragin et al. 2005), and in presurgical patients (Weiss et al. 2016a, b), spontaneous mesial-temporal lobe seizures with a hypersynchronous iEEG pattern are preceded by rhythmic FR on pre-ictal discharges with an incrementally increasing power. A similar pattern can also be seen during micro-seizures preceding low-voltage fast morphology seizures (Schönberger et al. 2019; Weiss et al. 2016a, b). A knowledge gap is that focal injury from kainic acid (Li et al. 2018; Sheybani et al. 2018, 2019) or TBI (Li et al. 2021b) can produce FR-generating brain regions far from the site of injury, yet not all these FR-generating sites independently generate seizures. Thus, our current understanding of PIN clusters is insufficient to discern the essential FR-generating sites for ictogenesis, and additional work is needed to extend the PIN cluster hypothesis for a widespread epileptogenic network as opposed to a narrow EZ.

5.5 Proposed Roles of FR in Surgical Planning

Complete resection of the SOZ does not always result in a seizure-free outcome (Fig. 5.2a) (Akiyama et al. 2011; Jacobs et al. 2010; Khan et al. 2022; Li et al. 2021a; Weiss et al. 2015), and ictal HFO could help more accurately delineate that brain area that needs to be removed to achieve seizure freedom (Bandarabadi et al. 2019; Weiss et al. 2013, 2015). However, using either seizures or ictal HFOs or both to plan a resection might not improve seizure outcomes because identifying all a patient's epileptogenic regions is constrained by the duration of monitoring time and the patient's number of habitual seizures. Interictal FR detected during sleep has been proposed as a better biomarker of the EZ (Frauscher 2020; Frauscher et al. 2017) and is used to calculate the FR resection ratio (RR, Fig. 5.2b) and FR graph theoretical metrics (Fig. 5.2c–g; and following section). An advantage of these metrics is they use the more abundant interictal episodes and can help better define all epileptogenic regions without capturing seizures. One study found that an FR RR of 60% identifies non-seizure-free patients with ~80% accuracy when using a 4-h sleep recording (Nevalainen et al. 2020). However, even in this study and in others (Haegelen et al. 2013; Jacobs et al. 2010, 2018), particularly one that utilized multi-center data (Jacobs et al. 2018), non-seizure free patients were misclassified because they had relatively high FR RR. Another problem with the FR RR is that it poorly handles spatial under-sampling by the iEEG. For example, if one brain site shows a very high-rate FR but two sites distant from each other contain moderate FR rates, then a resection that targets the very high-rate site results in a high FR RR. However, the territory between and around the other two moderate-rate FR sites may include unsampled, high-rate FR-generating regions. If a SEEG investigation poorly samples the EZ neither resection of the SOZ nor the FR RR will correlate with the

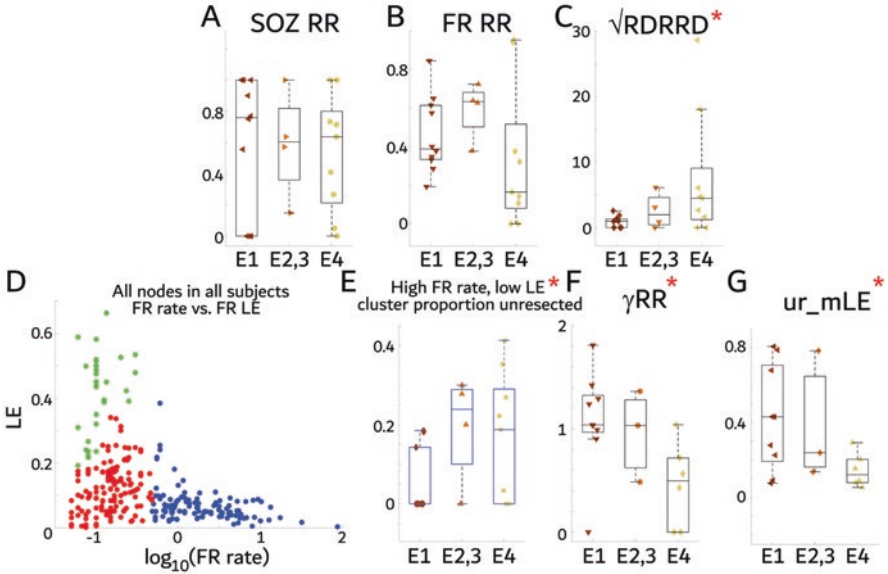


Fig. 5.2 FR graph metrics, but not the SOZ RR and FR RR, correlate with seizure outcome. (a–c) Box plots of the SOZ RR (a) FR RR (b) and the spatial FR network measure RDRRD (c) for each patient ($n = 23$) stratified by Engel (E) outcome. Red asterisk indicates a significant correlation with outcome (Spearman rank correlation, $p < 0.05$). (d) Scatter plot of the k-means clustered FR rate versus LE of each node from the FR MI network of all the patients, the nodes with zero LE are not shown. Red, green, and blue colored nodes correspond to the three defined clusters. (e) Box plot of the proportion of nodes in the high FR rate and low LE cluster (blue) left unresected for each patient and stratified by Engel outcome. (f, g) Box plots of the temporal correlational FR MI network measures γRR and ur_mLE for each patient with a complete FR MI network stratified by Engel outcome

outcome and this scenario may correspond with few predominant FR-generating regions (Nevalainen et al. 2020). Another limitation of the FR RR is that it is difficult to use prospectively to plan a resection/ablation (i.e., resection) since it does not specify what specific portion of the FR sites should be resected. An alternative strategy to predict the outcome is to identify the sites generating the relatively highest rate of FR and then ask if these sites were completely resected (Akiyama et al. 2011; Dimakopoulos et al. 2020; Fedele et al. 2017; Weiss et al. 2021). Researchers have found that this strategy is best applied to FR that are specifically superimposed on ripples (FR + R) (Dimakopoulos et al. 2020; Fedele et al. 2017). However, this strategy is not perfect because it is unclear whether the patient’s seizure outcome resulted solely from resecting the FR + R territory, which is often much smaller than the total resection volume (Weiss et al. 2021), or resecting the other neighboring brain tissue. Thus, the role of such a method in prospective surgical planning is limited.

5.6 Utilizing FR Graph Theoretical Metrics to Assess the Epileptogenic Network for Surgical Planning

In graph theory applied to EEG studies, a weighted graph consists of nodes (i.e., electrode contacts) connected by weighted edges, which can be computed, for example, from the correlation, coherence, or phase interactions between frequency bands or events in EEG signal (Bullmore and Sporns 2009; Rubinov and Sporns 2010; Sporns et al. 2005). A spatial FR graph measure called the rate-distance radius resection difference (RDRRD) is derived by calculating edge weights between interictal FR-generating nodes as the Euclidian distance between the nodes multiplied by the mean FR rate of each node. The radius of the network is an estimate of the FR-generating tissue weighted by rate. The difference between the whole network and the resected network radius is an estimate of the residual FR-generating tissue activity (Weiss et al. 2022a). We found that the RDRRD correlated with the outcome, but the SOZ RR and the FR RR did not (Fig. 5.2a–c) (Weiss et al. 2022a). The RDRRD also trended towards greater accuracy than the FR RR in predicting seizure-free patients, and more specific in predicting seizure-improved patients (Weiss et al. 2022a). Therefore, the RDRRD reduces misclassifications as compared to the FR RR likely due to overcoming the aforementioned spatial sampling limitations.

Two of our prior studies derived interictal FR temporal correlational networks using edges weighted by FR mutual information (MI), a non-linear measure of coherence or correlation, and calculated each node's local efficiency (LE) (Weiss et al. 2022a, b, 2023). In this approach, clustering nodes according to their LE and mean FR rate (Fig. 5.2d) (Weiss et al. 2022a, b, 2023) shows a higher proportion of unresected nodes with the highest FR rate and lowest synchrony (i.e., lowest LE) correlates with poor seizure outcome (Figs. 5.2d, e and 5.3). LE is proportional to synchrony because it is the inverse of the shortest average path length of a neighborhood around a single node. The path length is determined by the distance ($1/MI$) of the sequences of edges between nodes (Bullmore and Sporns 2009; Rubinov and Sporns 2010; Sporns et al. 2005). γ is the average shortest path between nodes in the entire network (Bullmore and Sporns 2009; Rubinov and Sporns 2010; Sporns et al. 2005). We derived one FR MI graph metric γ_{RR} by calculating γ in the resected territory network and γ in the whole brain network and measuring the RR. We found worse outcomes were associated with a lower γ_{RR} (Figs. 5.2f and 5.3) (Weiss et al. 2022a), which suggests in patients without seizure freedom, low LE sites were not resected. Another FR MI graph metric, ur_mLE is derived from the FR MI network left unresected and is the mean LE of the residual FR sites. We found lower ur_mLE was associated with a worse outcome (Figs. 5.2g and 5.3). These findings are in accord with the epileptogenic network hypothesis (Davis et al. 2021; Spencer 2002; Weiss et al. 2022a) and less supportive of the EZ hypothesis (Jehi 2018; Rosenow and Lüders 2001). In contrast to prior proposed notions of the epileptogenic network, which emphasize hubs of communication in seizure genesis (Coito et al. 2015, 2016; Lee et al. 2018; Royer et al. 2022), these results also demonstrate that

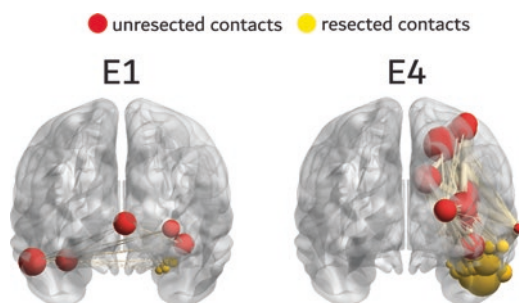


Fig. 5.3 Example braingraphs showing that in patients with Engel 1 (E1) outcome resected contacts exhibit lower fast ripple (FR) mutual information (MI) network local efficiency, and unresected contacts a higher FR MI network local efficiency. Braingraph of two patients with E1 (left) and E4 (right) outcome. The resected nodes are colored yellow, and unresected nodes are red. The size of the node corresponds to local efficiency. The edge size corresponds to the FR MI value

the most important regions are the highly active and asynchronous FR sites that generate epileptiform activity akin to the hottest, wildly burning, embers embedded deep in a fire.

It is anticipated FR temporal correlational graph theoretical measures could be utilized more effectively than FR RR or resection of FR + R regions in planning epilepsy surgery. Prospective “virtual resection(s)” (Jirsa et al. 2017; Khambhati et al. 2016; Kini et al. 2019) that target the high FR rate low LE nodes can be planned. Then, the projected outcome of the proposed “virtual resections” can be derived by applying machine learning using the FR graph metrics of the “virtual resection volume” as factors.

5.7 Stimulation Therapy of the Epileptogenic Network

Placement of the RNS device was initially thought to reduce seizure frequency by stimulating during ictal epochs and aborting the seizure (Lesser et al. 1999). However, the RNS device stimulates the brain over 1000 times a day and almost entirely during interictal periods (Razavi et al. 2020). Seizure frequency decreases gradually over the years following RNS (Nair et al. 2020). One study found that probable instances of ictal inhibition did not correlate with improved clinical outcome, but changes in the iEEG occurring remotely from the stimulation did correlate with the outcome (Kokkinos et al. 2019). Another study shows a reduction in seizure frequency following RNS placement correlates with reduced low-frequency iEEG connectivity measured over 1–3 years after implant (Khambhati et al. 2021). Thus, the efficacy of RNS may be more strongly related to induced alterations in the epileptic network (Khambhati et al. 2021; Piper et al. 2022). Furthermore, closed- and open-loop stimulation have been shown to be similarly effective (Vassileva

et al. 2018). A graph metric derived from the pre-surgical iEEG could predict RNS outcome (Scheid et al. 2022), but results are inconsistent with other published work (Kini et al. 2019) (and see preprint) (Scheid et al. 2021). In this latter work, it is hypothesized the RNS mechanism of action is to modulate FR networks and reduce seizures because of the intrinsic role of FR in epileptogenicity and ictogenesis (Bragin et al. 2000, 2002a, 2004, 2005). We have previously found that patients with seizure improvement after RNS placement have a narrow FR network with less active, asynchronous nodes than patients not offered an RNS because of widespread SOZ and non-responders to resection (Weiss et al. 2022a, b, 2023).

Our recent investigation of ten RNS patients found that three were RNS super responders (greater than 90% reduction in seizures (Khambhati et al. 2021)) and the other seven were intermediate responders (50–90% reduction in seizures) (Weiss et al. 2022b). The RNS was placed at least 4 years prior to outcome determination. We defined the pre-implant SEEG electrode stimulated contacts as within a radius of <1.5 cm of the eight RNS contacts (i.e., two leads of either a four-contact depth or subdural strip). Our justification for using a threshold of <1.5 cm was that RNS stimulation intensities typically range from 2–3 mAmps, and if the stimulation is monopolar, this corresponds to an electric field of 2–3 mV/mm at 1.5 cm away from the stimulation source (Plonsey and Barr 2000). Electric field strengths of at least 2–3 mV/mm can induce spike field coherence (Anastassiou et al. 2011; Ozen et al. 2010), and smaller fields at distances greater than 1.5 cm may only influence spike timing (Francis et al. 2003; Radman et al. 2007).

In the aforementioned RNS study, we determined the proportion of the pre-RNS SEEG electrode contacts deemed part of the SOZ also met our criteria of stimulated contacts and stratified this stimulation ratio (SR) by RNS outcome. We found that the 3 super responders exhibited a trend towards larger SOZ SR (Figs. 5.4a and 5.5). To test our hypothesis that RNS super responders have a less widespread FR network we calculated the FR SR, which is like the FR RR but uses stimulated rather than resected contacts. Stratifying the FR SR by the outcome we found that the super responders had significantly higher FR SR (Figs. 5.4b and 5.5). Also, we assessed if in super responders RNS, stimulation targets asynchronous FR sites, constructed FR MI networks including only the stimulated contacts, and calculated the global efficiency (Ge). This FR SGe, when stratified by the outcome, was significantly smaller in the super responders (Figs. 5.4c and 5.5) indicating in the three super responders, RNS leads were targeting highly active, asynchronous sites (Fig. 5.2d).

5.8 Summary

Pathological HFO includes FR without or with EEG spikes, especially FR containing power at high spectral frequencies, and some ripples on spikes or ripples coupled with the DOWN state. Pathological HFO could be generated from the abnormal spike firing of one or more groups of neurons, chiefly clusters of principal cells with

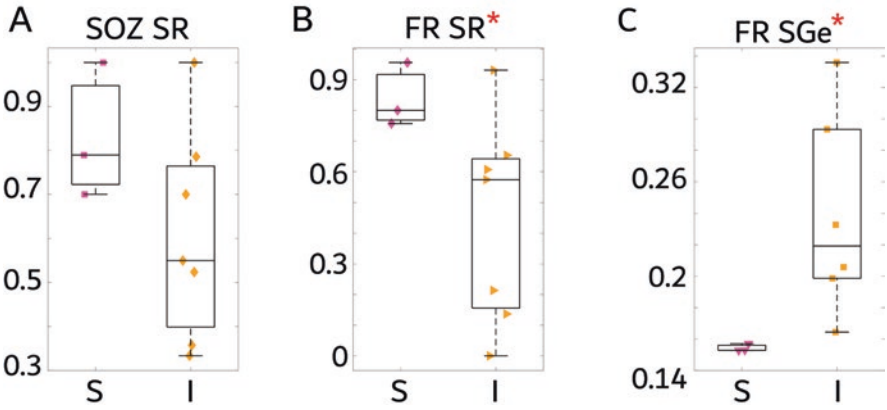


Fig. 5.4 In the cohort of 10 responsive neurostimulation (RNS) patients, RNS stimulation of FR sites correlates with super responder (S) seizure outcome status better than RNS stimulation of the SOZ. (a–c) Box plots of the SOZ stimulation ratio (SR, a), FR SR (b), and the temporal correlational FR MI network measure global efficiency of the stimulated FR network (SGe, c) for each patient stratified by RNS seizure outcome (S, super responder; I, intermediate responder). Red asterisk indicates a significant correlation with outcome (Spearman rank correlation, $p < 0.05$). The stimulated electrode contacts were defined as within 1.5 cm of the RNS stimulation contacts (see text)

alterations in interneurons that subtly decrease hyperpolarizing or paradoxically increase depolarizing postsynaptic potentials. In experimental models, it appears these neuronal disturbances can generate pathological HFO after an epileptogenic injury, their spatial distribution correlates with the subsequent appearance of spontaneous seizures, and coordination of pathological HFO-generating sites could facilitate the onset and spread of seizures after epilepsy is established. In clinical epilepsy, surgical removal of ictal and interictal correlate with good seizure control, but the ratio of FR removed in predicting seizure outcome could be prone to error due to constraints of extraoperative recording. Graph theory measures might overcome these constraints by more accurately representing the temporal and spatial properties of the FR and identifying essential FR-generating sites in the epileptogenic network that need to be removed for seizure freedom. Graph theoretical measures of FR in patients who receive RNS also might help identify FR-generating sites that need to be stimulated to control seizures.

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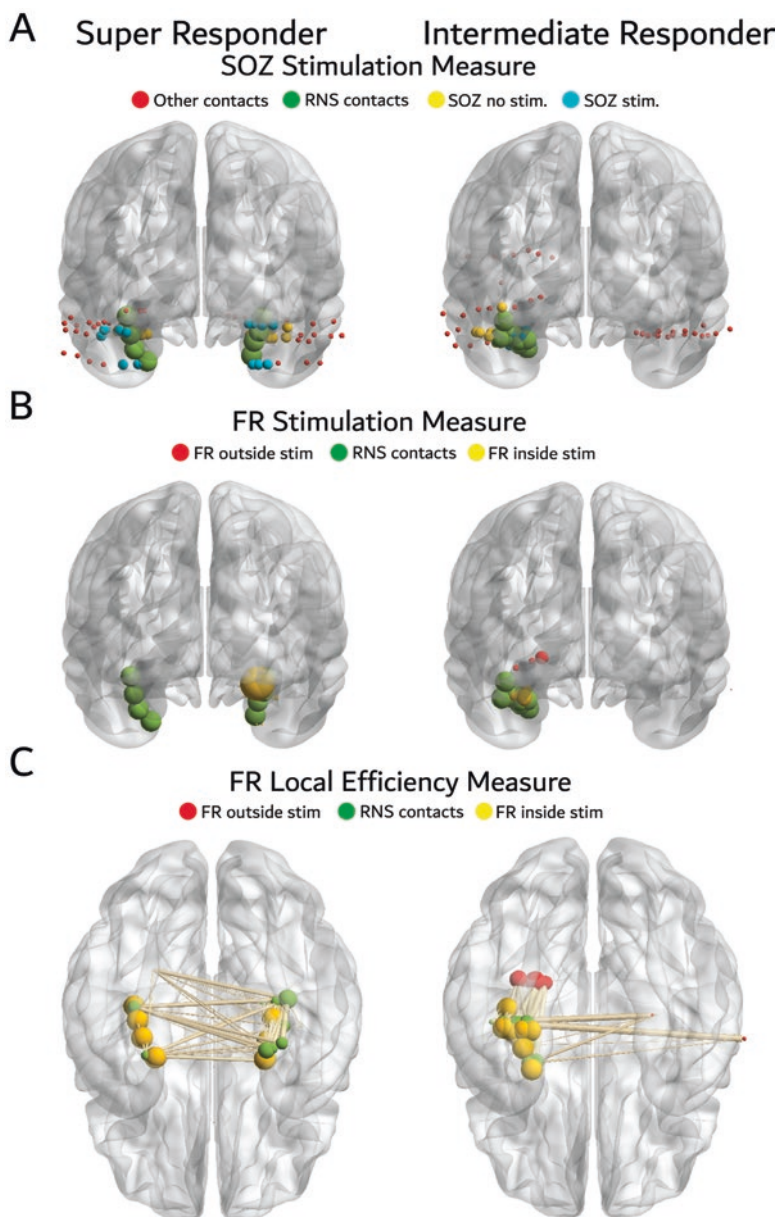


Fig. 5.5 Braingraphs of the pre-surgical iEEG contacts and responsive neurostimulation (RNS) contacts (green) illustrate the stimulation of fast ripple (FR) sites predicts RNS super responders. (a) The example super responder and intermediate responder have a relatively similar ratio of SOZ contacts that are stimulated (cyan) and unstimulated (yellow). (b) Stimulated FR generating nodes (yellow) and unstimulated FR generating nodes (red), the size of the node corresponds to the relative proportion of FR events generated by that node. The example super responder has exclusively stimulated FR nodes. (C) Stimulated (yellow) and unstimulated (red) FR nodes in this case the size of the node corresponds to the local efficiency (LE) of the stimulated FR network. The edge size corresponds to the FR mutual information (MI) value. Super responders have relatively lower FR LE and lower FR MI

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Chapter 6

Transporter Hypothesis in Pharmacoresistant Epilepsies: Is it at the Central or Peripheral Level?



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Abstract The multidrug resistance (MDR) phenotype characterizes patients with refractory epilepsy (RE). Seizures are not controlled despite receiving various combinations of more than two anti-seizure drugs (ASMs). The continued design of new ASMs did not change the constant percentage (30–40%) of epileptic patients who will develop the MDR phenotype. Drugs ASMs biodistribution, including their metabolites, depends on the functional expression of several transporters of the ABC transporters (ABC-t), P-glycoprotein (P-gp), the protein associated with resistance to multidrug (MRP-1) and breast cancer-resistant protein (BCRP). These transporters are constitutively expressed intestine, liver, kidney, and blood-brain barrier, playing a central role in pharmacokinetic balances. ABC transporters can be induced by stressful stimuli such as hypoxia, inflammation, the drugs administered, and even seizures themselves. Consequently, uncontrolled seizures increase the risk of RE by inducing greater functional expression of these transporters. Based on clinical and experimental findings, the so-called “transporters hypothesis” arises, which explains the MDR phenotype in ER, even when ASMs are administered simultaneously. These stimuli induce the ABC-t expression in cells that normally do not express them, such as neurons and cardiomyocytes, producing membrane depolarization that favors epileptogenesis, and heart failure, respectively, increasing the risk of developing sudden unexpected death in epilepsy (SUDEP).

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6.1 Introduction

About 50% of patients with epilepsy achieve complete seizure control with a first MSA (monotherapy), and a further 13% achieve remission with different combinations of two ASMs. However, a constant 30–40% of patients will remain with non-controlled seizures. S. M. Sisodiya (2005) proposed that as new ASMs appear aimed at blocking epileptogenic mechanisms not yet described, many patients considered resistant to drugs could no longer be so, suggesting that the appropriate drugs for treatment to patients with drug-resistant epileptic are not yet available (Sisodiya SM, 2005).

Since the use of bromide in 1860 as the first ASM, A significant number ($n > 20$) of ASMs have been incorporated into the epilepsy therapeutic arsenal. Despite the more specific, safe and effective effects of these new compounds, the LADME system (liberation, absorption, distribution, and excretion of drugs) will govern the final drug load that enters the CNS (Fig. 6.1)

From a clinical point of view, patients with refractory epilepsy present a phenotype characterized by resistance to multiple drugs called the “*MDR phenotype*,” and they will continue without adequate seizures control. Given that this percentage of drug-resistant patients has remained unchanged over time, it is imperative to find pharmacological strategies capable of defeating and/or avoiding the mechanisms responsible for the *MDR phenotype*. The most outstanding feature of the “transporter hypothesis” is that it is based on the inducible capacity of the ABC-t involved and, therefore, the increase in its vascular endothelial cells (VEC) level and the feet-end-processes of astrocytes, which surround the capillaries of the blood-brain barrier (BBB), that plays a key role limiting the access of the ASM to the central nervous system (CNS) (Tang et al. 2017; Pérez-Pérez et al. 2021).

In this chapter, we develop the evidence that shows, on the one hand, that the ABC-t generates pharmacokinetic changes in the ASM, and therefore, we consider these changes as peripheral mechanisms of drug resistance. On the other hand, we also describe the mechanisms of action of ABC-t (particularly P-gp) that have a potential epileptogenic role when expressed in neurons, and we consider this situation as a central mechanism responsible for drug resistance. The most distinctive feature of P-gp, initially described by Victor Ling, is its ability to be inducible in the membrane of tumor cells (previously P-gp negative), which confers resistance to a wide spectrum of different drugs that had never even been exposed to these cells (Ling 1989; Juliano and Ling 1976).

For what reason can a condition that is sensitive to drug treatment become a drug-resistant condition? Phenotypic modifications are the consequence of factors that simultaneously stimulate and/or repress the expression of different genes

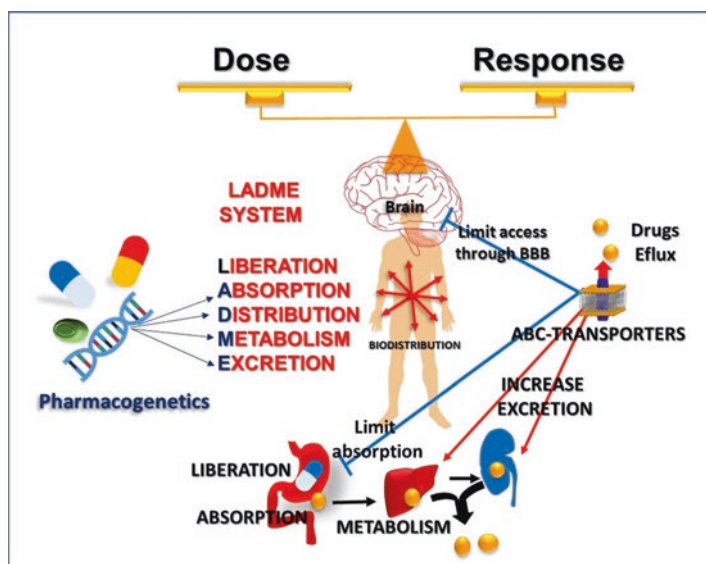


Fig. 6.1 The functional expression of ABC transporters in excretory organs and Blood Brain Barrier (BBB) can modify the concentrations of ASMs both in CSF and in plasma. The LADME system includes phase I enzymes such as the cytochrome P450 superfamily (P450s) responsible for 70% of the metabolism of all drugs and phase II glucuronidation for their subsequent excretion by transport systems (ABC-t). Interindividual differences in all these systems are governed by a wide range of genetic polymorphisms. (CSF cerebrospinal fluid)

related to the pharmacological response, and the seizures themselves can induce these changes (Auzmendi et al. 2021). This means that the longer it takes to bring seizures under control, the more likely it is that patients who might initially respond will become drug resistant due to changes in gene expression induced by the cumulative burden of seizure stress itself.

The correct choice of ASM, its adequate dosage, and frequency of administration are key to a balance between the administered doses, the concentrations of the circulating ASMs (blood and CSF), and satisfactory seizures control. On the contrary, lack of compliance by the patient, insufficient doses, and drug–drug interactions are the causes of decreased plasma levels of ASMs and, consequently, a reduced amount of ASMs accessing CNS that will be in equilibrium with plasma concentrations regardless of the route of administration (Rabinowicz et al. 1997; König et al. 2013).

Although the BBB exerts control of such access, changes in “peripheral” pharmacokinetic (PK) conditions induce an imbalance between the amounts of drug absorbed (decrease) and excreted (increase) and will affect the final circulating concentration of ASMs with a lower amount of drugs entering the CNS. In short, the overexpression of ABC-y in excretory organs and in BBB should be considered as peripheral pharmacokinetic changes related to drug resistance to ASMs.

Instead, the induction of ABC-t expression at the brain parenchymal cells, particularly neurons, should be considered as “central pharmacodynamic mechanism” of drug resistance to ASMs.

So, in sum, due to these dual (peripheral and central) roles of the ABC-t or both simultaneously, their functional inhibition could be part of the reversal of the phenotype). MDR in patients with RE was previously suggested (Robey et al. 2008).

6.2 The Multidrug Resistance (MDR) Phenotype

A highly surprising property of the “MDR phenotype” mediated by ABC-t is characterized by the ability of these transporters to extrude compounds directed to different therapeutic targets and with distinct chemical structures. This property of ABC-t, initially described in cancer cells, was also extended to other nonneoplasia eukaryotic cells and to the orthologues ABC-t expressed in microorganisms such as bacteria and yeast. More than 100 members of the ABC-t are part of a highly conserved superfamily in nature, of which 48 are found encoded in the human genome and divided according to structural similarities into seven different families (A–G), and only three have been identified associated with the MDR phenotype (Dean et al. 2001). Furthermore, they can confer 1000-fold or greater levels of drug resistance to cells that express them compared to cells that do not express them (Srikant 2020). The functional similarity and substrate overlap of these three ABC-t types (P-gp, BCRP, and MRPs) suggest both complementary and redundant roles (Gottesman and Pastan 1993). P-gp transports neutral or positively charged hydrophobic compounds and drugs without metabolic modifications, while members of the MRP and BCRP transporter subfamily transport mainly organic anions and phase II metabolic products (Dean et al. 2001) (Fig. 6.2). This indicates that the sum of expression of these three transporters allows the excretion of all kinds of natural compounds or drugs, whether they are cationic, neutral, or anionic compounds.

6.3 Role of ABC-t in the “*LADME System*” as the Peripheral Mechanism of Drug Resistance in Epilepsy

The functional expression level of different genes that govern biological processes such as drug absorption, distribution, metabolism, access to the brain, and excretion (LADME System) will be responsible for the modifications of equilibrium between the doses of ASMs administered, with both circulating concentrations and the specific concentrations at its site of action. Excretory organs, such as intestine, liver, and kidney, and biological barriers such as the BBB, are characterized by the presence of cell layers with a high polarized expression of ABC-t. In this way, they achieve the unidirectional excretory transfer (from the inside to the outside of the

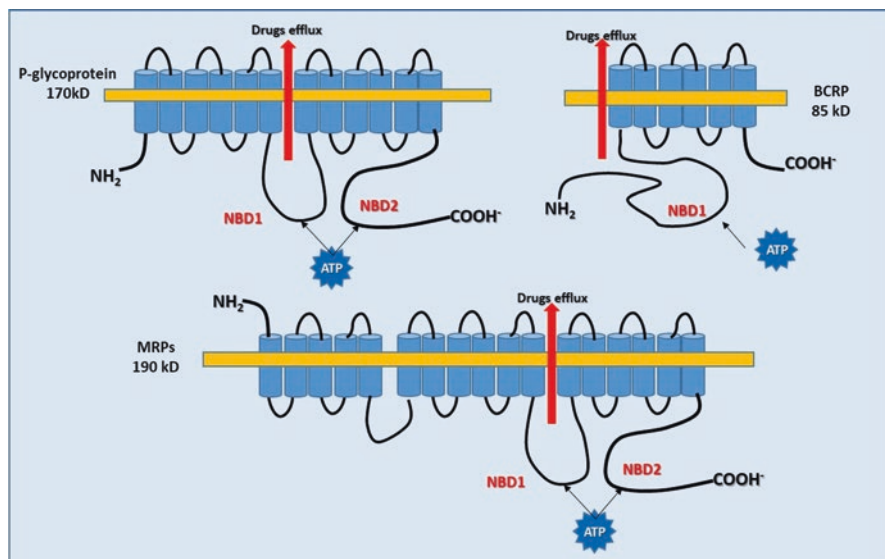


Fig. 6.2 Schematic structures of P-gp, MRP1–7 with longer NH₂ terminal chain and BCRP (hemimolecule); (ABC: ATP-binding cassette)

organism) of their different substrates, among them the ASMs. The expression of the ABC-t in the different excretory organs and in the BBB constitute a real “chemoprotection network” of the organism to avoid the toxic accumulation of different xenobiotic compounds or metabolic degradation (Sarkadi et al. 2006). There are interindividual differences in the functional expression of ABC-t in said organs, due to genetic variants or single nucleotide polymorphisms (SNPs), as well as secondary to the induction by a wide spectrum of inducing stimuli such as inflammation, convulsive crises, some nutrients, and the drugs themselves (antibiotics, antineoplastic, and ASMs).

The oral route is the most widely used route for the administration of most drugs, including ASM, which are normally well absorbed.

P-gp, MRP-2, and BCRP are the ABC-t found mainly in the apical membrane of the gastrointestinal tract (GIT). There they exercise the first step of controlling the amount of drug absorbed by producing the outflow from the interior of the enterocyte toward the intestinal lumen, reducing the amount of drugs that will reach the blood circulation. Subsequently, ASMs are metabolized by the liver through CYP-dependent oxidation phase I and conjugation phase II (glucuronidation or glutamylation). ABC-t expressed at the canaliculi level is responsible for the hepatobiliary elimination of metabolites and even the surplus of nonmetabolized drugs. The sum of the activities of the ABC-t and the hepatic metabolizing enzymes is a very efficient drug clearance system. Enzymatic and transport systems can be induced to be overexpressed or repressed because both mechanisms share some inducers, substrates, and inhibitors, playing complementary roles in controlling the

balance between administered doses and the amount of drug in blood circulation (Szakács et al. 2008).

In the LADME system, the CYP and glucuronidation enzymatic activities are limited to drug metabolism. Instead, the transporters are involved in three steps of LADME system, as absorption, biodistribution (including CNS access), and finally, drugs excretion (Szakács et al. 2008; Koehn et al. 2021) (Fig. 6.1).

It demonstrated the existence of a polymorphism in exon 26 (C3435T) of the *MDR-1* gene that encodes P-gp in Caucasian volunteers. In this study, individuals carrying the CC allele (homozygous) had elevated P-gp protein expression in the small intestine that was associated with low plasma levels of orally administered digoxin, compared with individuals carrying the TT allele (homozygous). Digoxin is a drug that does not undergo metabolic changes but is 100% transported by P-gp. Additionally, the administration of rifampicin (an inducer of P-gp glycoprotein expression) increased its duodenal expression with a concomitant furthermore decreased digoxin plasma levels in CC subjects, but these effects were not observed in TT subjects (Hoffmeyer et al. 2000).

^{99m}Tc -hexakis-2-methoxy-isobutylisonitrile (^{99m}Tc -SESTAMIBI), like digoxin, is a compound that does not undergo any metabolic transformation and is 100% a substrate for P-gp (Fig. 6.3b). ^{99m}Tc -SESTAMIBI is a widely used radiotracer for single photon emission computed tomography (SPECT) studies. Through a SPECT study of the hepatic clearance of ^{99m}Tc -SESTAMIBI, it was possible to demonstrate that adult patients with ER ($n = 8$) presented accelerated values in comparison with normal controls and responding epileptic cases (Fig. 6.3a). Five of these eight RE

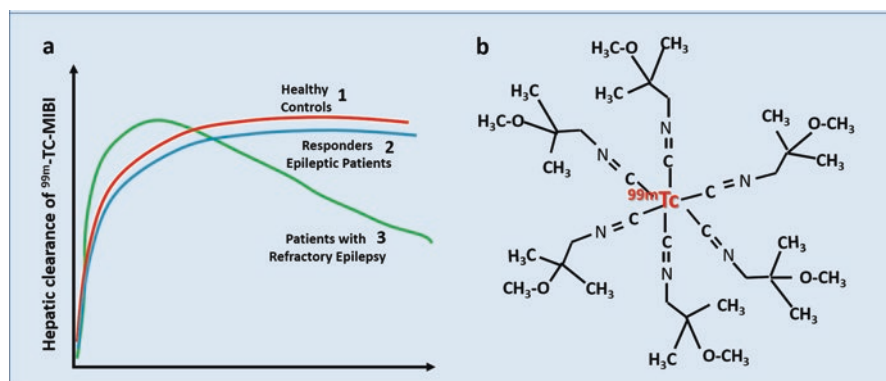


Fig. 6.3 (a) The hepatic clearances of ^{99m}Tc -SESTAMIBI were similar in healthy controls and responders epileptic patients seizure-free (1-red line and 2-blue line respectively), but much accelerated in RE patients (3-line red). In two cases with RE, and after administration of nimodipine (2 mg/kg), the hepatic clearance was recovered to similar values to healthy controls or responders patients. These findings suggest that in patients with uncontrolled seizures, the functional expression of P-gp in excretory organs may have a negative impact on the pharmacokinetics of ASM, increasing their excretion. (b) Molecular structure of ^{99m}Tc -SESTAMIBI (Vazquez et al. 2004; Czornyj et al. 2022)

patients were surgically treated, and in all of them, P-gp was immunodetected in the neurons from the epileptogenic brain area removed (Vazquez et al. 2004).

In Caucasian epileptic patients carrying the SNP C3435C of the MDR1 gene, phenobarbital (PhD) concentrations in CSF and serum were significantly lower than in heterozygous CT and homozygous TT patients (Basic et al. 2008). However, an opposite genotype-phenotype relationship was detected in Japanese cases (Seo et al. 2006), and 3435-TT homozygous Chinese RE patients had decreased plasma carbamazepine (CBZ) levels (Meng et al. 2011). More recently, a systematic review meta-analysis confirmed the 3435-SNPs of MDR1 gene inverse functionality in “orientals patients” (Chouchi et al. 2017).

We suggest that the pharmacogenetic evaluation of this polymorphism will be useful for a better adjustment of the ASM doses according to each ethnic group and accompanied by the corresponding measurement of the plasmatic levels of the drugs. So therapeutic monitoring of ASMs, makes it possible to detect persistent decreases in plasma levels, which may be more evident during long periods of hospitalization and associated with lack of seizure control but not related to noncompliance (Lazarowski et al. 1999, 2004a) or inversely, detecting their higher salivary excretion (Fagiolino et al. 2013) in patients with RE.

More recently, our group has also documented a high frequency of plasmatic persistent low levels (PPLL) of more common ASMs as PHT, PhB, VA, and CBZ, in a pediatric population of 3279 epileptic patients treated with two or more ASMs (polytherapy). In this study, we analyzed retrospectively 21,040 results of plasmatic concentrations of the mentioned ASMs. The PPLLs of PHT were detected in 71.7% of inpatients and 74.1% of outpatients from this population. In the other ASMs, the PPLLs were also detected in both inpatients and outpatients, but in a smaller proportion. In some patients, PPLL of at least one ASM during long periods of hospitalization was documented as a dominant feature for four drugs evaluated (Czornyj et al. 2018). All these results suggest that the PPLL of ASMs, detected particularly during hospitalization periods, can be interpreted as a laboratory error or attributable to poor quality of the ASMs and, therefore, can induce withdrawal of the drug found with PPLL in plasma. However, they should be considered as a sensitive biomarker of pharmacokinetic changes related to critical clinical conditions of patients.

In other studies that gathered 70 epileptic patients under treatment with oral doses of PHT, the plasma concentration of the free fraction of PTH was significantly diminished in drug-resistant patients than in responder's epileptic patients (Iwamoto et al. 2006). Based on these and other similar results, it has been suggested that the synergistic increase in enzymatic activity (metabolism) and transporters (excretion) can reduce plasma levels of ASM, which gave rise to the so-called “pharmacokinetic hypothesis” of drug resistance in epilepsy (Tang et al. 2017).

In this sense, it is important to point out that cannabidiol (CBD) is not only an inhibitor of P-gp output activity in the cells of the neurovascular unit (Auzmendi et al. 2020b) but also inhibits some CYP isoforms, both mechanisms related to brain access and the PK of ASMs. Furthermore, the therapeutic use of CBD increased the plasmatic levels of several ASMs (Geffrey et al. 2015; Klotz et al. 2019; Socala et al. 2019; Ebrahimi-Fakhari et al. 2020). Regardless of its multitarget action, these

PK effects may partly explain the beneficial use of CBS as an adjuvant treatment in RE.

Interestingly, experimental chronic administration of PHT in rats induced a transient liver overexpression of P-gp that was reversed when PHT treatment finished (Alvariza et al. 2014).

Different stimuli involved in apoptosis, stress, inflammation, and hypoxia, such as hormones, oncogenes, and transcription factors such as p53, NFkB, IL6, AP-1, and HIF-1 α , can induce overexpression of the transporters at the excretory organs level, but also simultaneously can induce de-novo expression of ABC-t in previously nonexpressive cells such as neurons and cardiomyocytes (Auzmendi et al. 2014).

Repetitive without control seizures can induce P-gp expression in the brain parenchymal cells (neurons and astrocytes) and vascular endothelial cells of BBB.) (Lazarowski et al. 2004b) playing a “central role” which we will discuss below, but also in peripheral organs such as the liver and kidney, directly related to increased drug clearance (Guo and Jiang 2010). These peripheral impact of convulsive stress was also related experimentally to sudden unexpected death in epilepsy (SUDEP) in rats due to the seizure-induced P-gp expression in cardiomyocytes, which was associated with heart failure, bradycardia, long QT, and high ratio of spontaneous death (Auzmendi et al. 2014, 2018).

6.4 ABC-t in the Central Mechanism of Drug-Resistant Epilepsy

6.4.1 *Does the Expression of P-gp in Neuronal Membranes Play an Epileptogenic Role?*

In brain, P-gp is normally expressed only in brain vascular endothelial cells (VECs) of blood-brain barrier (BBB) and in the food-ending process of astrocytes (Verscheijden et al. 2020; Enrique et al. 2021). This normal expression plays a chemoprotective role in the brain; however, this expression could be downregulated under different conditions, reducing the cerebral protection as described in Parkinson’s disease and in stages of advanced neurodegeneration in Alzheimer’s disease (Bartels et al. 2008; Vogelgesang et al. 2002).

In contrast, in other studies developed in brain samples from epileptogenic brain areas surgically removed from adults patients with RE, increased levels of *MDR-1* gene transcripts as well as elevated P-gp immunoreactivity in both endothelial and glial cells were detected (Sisodiya et al. 2001, 2002; Tishler et al. 1995). Similar results were detected in dysplastic neurons and balloon cells, from a child with RE due to tuberous sclerosis as well as in a lot number of neurons in another report of

a drug resistance epileptic pediatric patient, both cases above mentioned with PPLL of ASMs (Lazarowski et al. 1999, 2004a).

Our group and other authors have also shown that not only P-gp but also MRP and BCRP were overexpressed in neurons from brain biopsy samples from pediatric RE patients. In certain cases, the presence of a cytoplasmic protein that sequesters drugs known as major vault protein (MVP) was also detected (Lazarowski et al. 2004c, 2006a, b; Brukner et al. 2021; Banerjee Dixit et al. 2017); in a pediatric case with focal cortical dysplasia (Czornyj et al. 2005, 2022), or with transmantle cortical dysplasia (Czornyj et al. 2021), and some patients with epilepsy due to brain tumors (Czornyj and Lazarowski 2014; Lazarowski et al. 2014). Similar results were also described in adult patients with temporal lobe epilepsy (Ak et al. 2007; Aronica et al. 2003a, b).

The neuronal P-gp expression has also been reported in different animal convulsive models (Volk et al. 2004; Lazarowski et al. 2004c) and also in different experimental brain hypoxia (Ramos et al. 2004; Lazarowski et al. 2007a, b; Aviles-Reyes et al. 2010; Merelli et al. 2011, 2019).

Experimentally, in a model of seizures resistant to PHT due to high expression of P-gp induced by mercaptopropionic acid, we were able to show that the use of nimodipine (2 mg/kg), which inhibits P-gp activity, restored the normal flow of PHT in the hippocampus with complete recovery of seizure control (Höcht et al. 2007). A distinctive feature of these experiments was that greater seizure severity evaluated by the “racine scale” with 100% resistance to PHT was only observed when a large number of neurons were P-gp-immunoreactive, and this abundant neuronal P-gp expression was also associated with the development of status epilepticus and a concomitant P-gp in cardiomyocytes (Auzmendi et al. 2014). This heart feature was later associated with bradycardia, long Q-T segment, a high rate of spontaneous death (Auzmendi et al. 2018), and more recently with heart ferroptosis with myocardial iron overload (Akyuz et al. 2021). These last cardiac complications associated with SUDEP are additional peripheral comorbidities where the ABC transporters play an important role in the depolarization of cardiomyocytes that will be extensively discussed in Chap. 11 (Fig. 6.4).

Because therapeutic targets of ASMs are expressed on the outer face of neuronal membranes, the P-gp efflux activity expressed at this same level, should not alter the pharmacologic action of ASMs. However, in our experiments, we observed that the maximum refractoriness was found when a high level of P-gp expression involved a large number of neurons.

So, What Is the Functional Role of P-gp Expressed in Neuronal Membranes?

We believe that this expression is not consistent with the classic function of drug transport/drug efflux, and so another functional role should be postulated (Fig. 6.5).

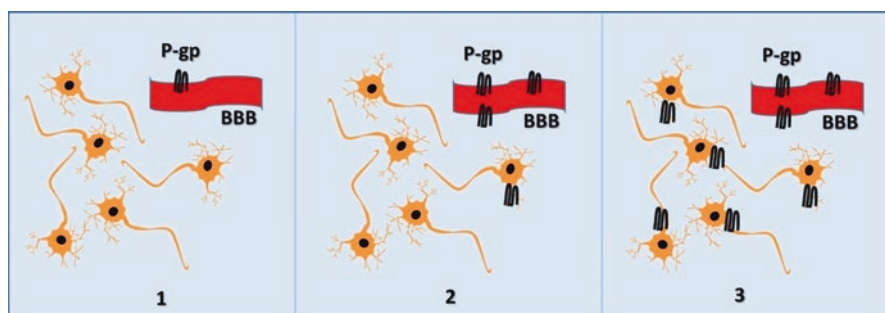


Fig. 6.4 Schematic representation of the progressive increase in neuronal P-gp expression as a result of the cumulative number of seizures induced daily. (1) Basal condition; (2) after 1 seizure-induced daily for 4 consecutive days associated with 50% of PHT resistance; (3) after 1 seizure-induced daily for 7 consecutive days, associated with 100% resistance to PHT, and a progressive worsening of the severity of the seizures

6.4.2 *ABC Transporters and Phosphatidylserine Translocation to the Outer Face of the Cell Plasmatic Membrane. A Potential Mechanism of Epileptogenesis*

Biomembranes, including neuronal membranes, function by separating extracellular electrical charges from intracellular ones. Different membrane properties such as fluidity, dielectric constant, and thickness can be modified by alterations in their lipid composition. These changes can induce modification on the membrane potential, increasing neurons' excitability. Furthermore, lipid composition changes may also result from transporters overactivity; however, this needs to be investigated in future studies, both in vitro and in vivo.

Phosphatidylserine (PS) usually comprises a low % (~3–5) of total phospholipids of mammalian cells, but its concentration is much higher (15–33%) in the inner leaflet of the plasma membrane.

Three families of proteins are involved in the transverse movement of lipids across membranes. They are the scramblases, some ATP-binding cassette (ABC) transporters, and the P4-ATPases. The last two use the energy of ATP hydrolysis, necessary for the translocation of lipids that guarantees their asymmetric distribution in biological membranes (Coleman et al. 2013).

P4-ATPases, are not ABC transporters; they act as phospholipid flippases transporting specific phospholipids from the extracellular face to the cytoplasmic leaflet of membranes to generate and maintain membrane lipid asymmetry (Andersen et al. 2016).

Inversely, it was reported that some ABC-t as ABCA1 and ABCB1 (P-gp) can act as floppases and translocate the lipids as phosphatidylserine from the inner face to the exoplasmic leaflet of membranes (Quazi and Molday 2013; Pohl et al. 2002).

However, the neuronal expression of the P4-ATPases complex (including the complementary CD50 subunit) was not reported in the literature yet. This apparent

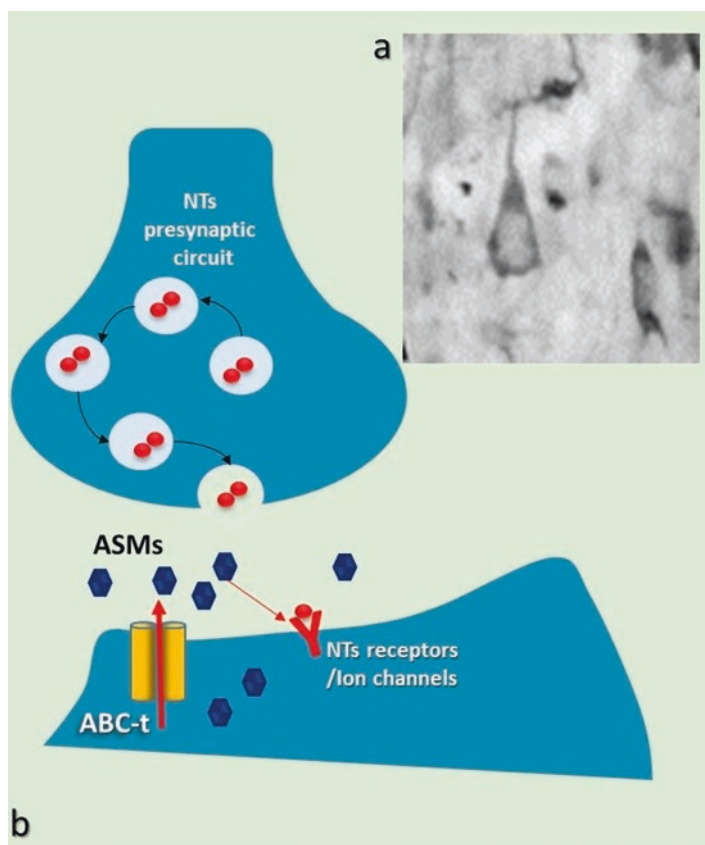


Fig. 6.5 (a) Neuron expressing P-glycoprotein in the soma; (b) The expression of P-gp in neuronal membranes should increase the concentrations of ASMs at the inter-synaptic space, helping their therapeutic effects. However, this P-gp neuronal expression was related to the most severe pharmacoresistant phenotype in both our experimental models and clinical cases (Lazarowski et al. 2004a, b; Czornyj et al. 2022). Consequently, we suggest another functional mechanism of P-gp explaining the MDR phenotype associated with this immunohistochemical finding (a)

absence of expression of P4-ATPases in neurons suggests that neurons may be unprotected against the potential activity of floppases that translocate PS to the outer face of the cell membrane.

Furthermore, similarly observed with ABCB1 (MDR-1 gene), it was reported that the hypoxia-inducible factor 1 (HIF-1) induces the ABCA1 gene expression (Ugocsai et al. 2010). In this regard, it was reported that P-gp activity could translocate PS to the external side of the plasmatic membrane (Dekkers et al. 1998; Pohl et al. 2002; Sugawara et al. 2005). All these evidences suggest that seizures, assumed as hypoxia-ischemia episodes, induce the expression of ABC-t with floppases properties. Consequently, a net negative charge will be generated on the outer face of the

neurons, which can reduce the convulsive threshold (epileptogenesis) and which, in turn, cannot be regulated with ASMs.

According to these concepts, it was also reported that phosphatidylserine decarboxylase (PSD) deficiency, with decreased Phosphatidylethanolamine and increased PS concentrations, is directly related to seizures. A class of *Drosophila* mutants that exhibit a “transient paralysis following a brief mechanical shock” was identified in a behavioral screen and reported in 1971 (Benzer 1971). Furthermore, it was suggested that unbalance of lipid components in biological membranes are directly related to several neurological illness, including epilepsy (Witt 2015).

Moreover, an experimental study demonstrated that beta-hydroxybutyrate (β Hb)-restricted ketogenic diet (KD) and decanoic acid modified lipid composition in HT22 murine hippocampal neurons *in vitro*. In turn, incubation with β Hb resulted in a decrease in PS levels, while the levels of other phospholipids remained almost unchanged (Dabke et al. 2020).

Interestingly, a recent report demonstrated that sebacic acid (SA), another KD component, displayed the best activity profile reversing resistance to phenytoin (PHT) and decreasing the P-gp upregulation in an experimental model of RE (Enrique et al. 2021).

There are two basic enzymatic activities that regulate the distribution of PS between the two leaflets. One is responsible for removing PS from the external leaflet by ATP-dependent active transport, and the most relevant protein playing this role is a member of the type IV subfamily of P-type ATPases (P4-ATPases) encoded by 14 genes (Bever and Williamson 2016).

On the other hand, within the family of ABC transporters, the ABCA1 is a cholesterol transporter that also acts as phosphatidylserine (PS) translocase, increasing PS levels in the outer face of the membrane (Smith et al. 2002). Because a number of tumor cell lines have been reported to express 3–7-fold elevated amounts of PS on the exoplasmic membrane leaflet compared with nontumorigenic cells, and these cells also overexpress ABCB1 (P-gp) displaying an MDR phenotype, it was suggested that P-gp could also translocate PS (Utsugi et al. 1991). Furthermore, the use of some inhibitors of P-gp as PSC 833, cyclosporin A, and dextraguidipine hydrochloride, inhibited the PS translocation in EPG85–257 human gastric carcinoma cells overexpressing P-gp (Pohl et al. 2002). Taken together, these data suggest that P-gp overexpression in neurons could induce the PS exteriorization, increasing a net negative charge on the outer face of the membranes, lowering the seizure threshold (Fig. 6.6).

In P-gp-positive tumor cells, an alternative mechanism to the well-known pumping function of the pump was described because these cells had a significantly low membrane potential ($\Delta\Psi_0 = -10$ to -20) compared to the physiological potential ($\Delta\Psi_0 = -60$ mV) (Hoffman et al. 1996; Wadkins and Roepe 1997). Based on these results, a possible role of P-gp in neurons could be to promote a greater membrane depolarization, thereby facilitating the development of new seizures and/or increasing their severity. We demonstrated in an experimental model of repetitive seizures induced by pentylenetetrazole (PTZ) that the overexpression of P-gp in the cerebral cortex and hippocampus contributes to the progressive depolarization of the cell

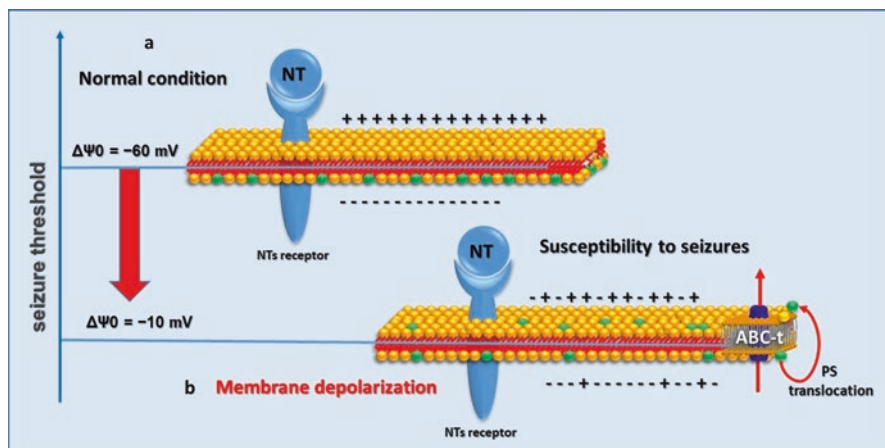


Fig. 6.6 (a) Schematic normal distribution of membrane phosphatidylserine at the internal face of the plasmatic membrane with normal physiological membrane potential ($\Delta\Psi_0 = -60\text{ mV}$). (b) The expression of P-gp or other ABC transporter could translocate PS increasing the negative charge on the outer side of the membrane, bringing the membrane potential to values between $\Delta\Psi_0 = -10$ to -20 mV . This change reduces the seizure threshold, which is an epileptogenic scenario. (NT: neurotransmitters; PS: phosphatidylserine (●))

membranes. This condition was associated with PHT-pharmacoresistant seizures, and only the combined administration of PHT plus nimodipine restored normal membrane potential and seizure control (Auzmendi et al. 2013).

However, the intimate mechanism of this depolarizing property of P-gp, observed in both tumor cells and brain tissue sections, has not yet been elucidated. Seizures could induce P-gp expression through different mechanisms. In the early 1970s, cerebral hypoxia was described as causing seizures (Madison and Niedermeyer 1970).

In eukaryotic cells, hypoxia is a condition that can induce mechanisms of rescue and survival or several mechanisms of programmed death, both depending on the intensity and/or durability of hypoxic stress. These differential dual responses are mediated by factor HIF1 α (hypoxia-inducible factor 1- α). HIF1- α was discovered by Greg L. Semenza, who received the 2019 Nobel Prize in Physiology or Medicine for demonstrating how the body's cells sense and react to low oxygen levels. HIF-1 α is known as the master of transcription factors, playing an important role in stimulating or repressing an extensive list of genes that modify the functionality of cells under oxygen deprivation (Semenza 2017).

Under hypoxic conditions, genes encoding erythropoietin (EPO) and erythropoietin receptor (EPO-R) are stimulated by HIF-1 α (Merelli et al. 2011). P-gp can be induced by different mechanisms, among them by seizures through glutamate signaling and activation of the cyclooxygenase-2 pathway (Bauer et al. 2008), but also by hypoxia itself in a HIF-1 α dependent manner (Comerford et al. 2002).

We have shown that neurons can express both P-gp and EPO-R, not only under hypoxic conditions but also after convulsive stress, in both situations, accompanied by a translocation of HIF-1 α at the nuclear level. Furthermore, EPO administration

not only protected against ischemic brain damage *in vivo* but also inhibited P-gp-mediated Rho-123 transport *in vitro* (Lazarowski et al. 2007a, b; Merelli et al. 2011, 2019). These results not only indicate that the convulsive phenomenon leads to a hypoxic condition (discussed in depth in Chap. 11) but also support the therapeutic opportunity of using EPO as therapeutics in hypoxic brain conditions and RE with P-gp overexpression (Lazarowski et al. 2007a, b; Merelli et al. 2011, 2019).

6.5 Brain Inflammation, ABC-t, and Blood-Brain Barrier Dysfunction

Brain trauma, cerebral hypoxia, and seizures share various sequential mechanisms, beginning with excitotoxicity, then depolarization, inflammation, and ending with programmed neuronal death (apoptosis or ferroptosis) (Merelli et al. 2021). In all cases, immune activation and inflammation, collaborate with the pathogenesis of epilepsy, with active participation of different inflammatory mediators such as interleukins IL-1 β , IL-6, tumor necrosis factor- α (TNF- α), also accompanied by oxidative stress (Vega-García et al. 2021; Lorigados Pedre et al. 2013), and by a wide spectrum of altered mechanisms of the BBB function (Marchi et al. 2012). These alterations include an induced overexpression of P-gp (Lazarowski et al. 2007a, b), dysfunction of glucose transporter GLUT1 with decreased brain glucose uptake in the epileptic area (Janigro 1999), subcellular mislocalization of AQP4 that significantly affects neuronal hyperexcitability, playing an epileptogenic role (Szu and Binder 2022). AQP4 is normally expressed at the astrocyte end feet associated with the BBB. However, in the sclerotic hippocampus, its expression in BBB is reduced but increased at the brain parenchymal cells. This translocation contributes to water leakage and impaired K⁺ buffering (Strohschein et al. 2011), which is dependent on Kir 4.1 expression, a potassium channel that is reduced in epileptic brains (Marchi et al. (2012). These modifications are a consequence of the stressful insult and may be transitory as long as the initial insult is not repeated. So, intracerebroventricular injection of kainate induced a transient expression of P-gp in astrocytes, and the immunostaining was detected up to 10 weeks postinjection (Zhang et al. 1999). Transient P-gp expression in neurons was reported for the first time in an experimental model of partial brain ischemia by cortical revascularization in rats. This procedure induced a maximum P-gp expression in cortical neurons at day 7 postinjury, which reduced to undetectable levels by day 28 (Ramos et al. 2004).

These results indicate that the duration of the induced expression of P-gp is limited in time, depending on the intensity of the stimulus and the type of expressing cell. In turn, they suggest that the window of drug resistance will depend on the intensity and repetition of the inducing stimulus but that it can be reversed if the stimulus is annulled. On the other hand, if the initial insult is repeated in the short term, it will generate a sustainable expression over time, giving chronicity to its drug-resistant function. Consequently, repetitive inducing insults are expected to increase long-lasting P-gp expression as a window of therapeutic resistance.

In this sense, it is important to highlight the recent advances in the use of cannabinoids for the treatment of RE (Farrelly et al. 2021). While several mechanisms have been proposed by which cannabinoids might exert their beneficial effects for more severe types of epilepsy (Friedman and Devinsky 2015; Rocha et al. 2020), it was also demonstrated a direct inhibitory effect of cannabinoids on COX2 activity (Takeda et al. 2008), playing a role not only as anti-inflammatory agents but also blocking an important pathway of P-gp induction (Zibell et al. 2009). More recently, our group was able to show a direct effect of cannabidiol (CBD) on P-gp function by detecting through coupling studies that CBD has two binding sites on P-gp, exercising a strong inhibitory effect on the exit transport of the substrate Rho-123, in cultured P-gp positive cells (Auzmendi et al. 2020a, b), such as that induced by nimodipine (Höcht et al. 2007). These findings provide a molecular basis that partly explains the efficacy of the adjuvant use of cannabidiol together with ASMs for better control of seizure in RE. (Lattanzi et al. 2021).

6.6 Interconnection of Central and Peripheral Role of ABC Transporters in Refractory Epilepsy and SUDEP

6.6.1 *Expression of P-Glycoprotein in Cardiomyocytes and Its Potential Role in SUDEP Development*

Sudden unexpected death in epilepsy (SUDEP) is the death of a person with epilepsy that is not caused by injury, drowning, or other known causes and is an important concern for people with RE who have a higher risk than epileptic responders patients (Devinsky 2011). It has not yet been possible to identify biomarkers capable of announcing this fatal outcome. However, a growing consensus indicates that acute heart failure can be the main cause of SUDEP. Furthermore, this heart failure could be the consequence of excessive hypoxic stress induced by repeated and severe convulsive crises that produce Ictal Hypoxia (HI). Added to this stress is neurocardiogenic injury due to sympathetic overstimulation. Together, both mechanisms would have a direct correlation with severe cardiorespiratory alterations and SUDEP. (Bruno et al. 2018). Therefore, it is not surprising that the main risk factor for SUDEP is the severity and frequency of generalized tonic-clonic seizures (GTCS). An emerging concept that has gained great interest was recently named as “Epileptic Heart” and described as “a heart and coronary vasculature damaged by chronic epilepsy due to repeated hypoxemia and increased catecholamines leading to electrical and mechanical heart dysfunction” that is characterized by heart failure with malignant bradycardia (fatal arrhythmia) (Verrier et al. 2021, 2022a, b).

Just as we suggest a depolarizing role of P-gp expressed in neurons associated with epileptogenesis, we also believe that the de-novo expression of P-gp in cardiomyocytes plays a similar role, collaborating in the development of heart failure and, therefore, consequence in SUDEP. In this regard, let us remember that under normal conditions, P-gp is absent in cardiomyocytes and low expressed in the endothelial

cells of capillaries and arterioles in the healthy heart (Cordon-Cardo et al. 1990; Meissner et al. 2002). Seizures can induce P-gp expression in the brain (neurons, astrocytes, and VECs of BBB) and in the heart (cardiomyocytes). In both cases, this expression is associated with a concomitant activation of HIF-1 α . Consequently, the highly cumulative load of convulsive stress (as in repetitive GTCS in cases of RE and/or status epilepticus) results in a severe hypoxic cardiac insult (Auzmendi et al. 2017, 2020a, b), where P-gp heart expression would be part of the depolarizing mechanisms of cardiomyocytes (Auzmendi et al. 2018). Furthermore, decreased mRNA transcripts of KIR channel, which play a critical role in the repolarization of cardiomyocytes, was documented in heart, after PTZ-induced repetitive seizures (Akyüz et al. 2018). So, both mechanisms of P-gp induced expression and KIR reduced expression will collaborate in the persistent depolarization of cardiomyocytes membranes (Auzmendi and Lazarowski, 2020)

Heart rate is regulated by Kir potassium channels that modulate membrane potential in cardiomyocytes, and in this sense, different genetic variants of these channels have been related to epilepsy and cardiac dysfunction (Abraham et al. 1999). In Chap. 10, we develop the evidences indicating that repetitive seizures is associated with an acute fatal heart rhythm alteration as a result of the severe hypoxic stress mentioned.

6.7 Conclusions and Remarks

The overexpression of ABC transporters in the excretory organs and in the BBB plays a key role in limiting the absorption and entry into the CNS and increasing the elimination of ASMs.

In turn, as a consequence of repeated seizures, the induction of its expression in cells that normally do not express ABC-t, such as neurons and cardiomyocytes, can generate a depolarization of these cells associated with increased epileptogenic risk and cardiac dysfunction associated with SUDEP.

Based on all this evidence, we can state that cardiac disorders can induce alterations of the BBB, status epilepticus, seizures, and epilepsy, and conversely, uncontrolled ER seizures, and status epilepticus, can induce cardiac dysfunctions. This vicious circle was well defined by Friedman and coined as the concept of the “chicken and egg” puzzle, with a high risk of death (SUDEP) (Friedman 2011).

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Chapter 7

Changes in Targets as an Explanation for Drug Resistance in Epilepsy



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Abstract Drug resistance in epilepsy is a condition that limits the control of seizure activity. The drug target hypothesis postulates changes in those targets on which antiseizure medications act. Alterations in drug targets can be structural or localization in nature and signal transduction changes. This chapter describes changes in targets associated with drug resistance in epilepsy. In addition, other mechanisms are suggested by which target availability or signaling might be compromised, contributing to the pharmacoresistant phenotype of epilepsy.

Keywords Drug-resistant epilepsy · Target hypothesis · Receptors · Antiseizure medication · Desensitization · Downregulation · Lipid rafts · Signaling changes

7.1 Introduction

The drug target hypothesis indicates that the lack of efficacy of antiseizure medications (ASM) in drug-resistant epilepsy (DRE) is a consequence of structural or functional alterations of their targets (Remy and Beck 2006). This hypothesis is postulated based on the insensitivity of voltage-gated sodium channels (VGSC) to carbamazepine observed in the resected hippocampus of DRE patients and experimental models of epilepsy (Remy et al. 2003a; Vreugdenhil and Wadman 1999). This chapter discusses several mechanisms that support the drug target hypothesis in DRE, including altered receptor subunit expression, epigenetic changes, receptor mosaicism, and lipid raft expression.

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7.2 Voltage-Gated Sodium Channels

Voltage-gated sodium channels (VGSC) are transmembrane proteins activated by changes in the potential that allow sodium transportation along an electrochemical gradient when they open (De Lera Ruiz and Kraus 2015). In the resected cortex of patients with DRE, VGSC mRNA expression is upregulated, which may contribute to the increased neuronal excitability associated with epilepsy (Lombardo et al. 1996). In addition, evidence shows decreased mRNA and protein expression of the $\beta 4$ subunit of VGSC in hippocampal tissue resected from patients with DRE. Altered expression of the $\beta 4$ subunit could decrease the frequency and duration of the VGSC resting state and, thus, be implicated in the loss of ASMs efficacy (Sheilabi et al. 2020).

In the pilocarpine-induced epilepsy model, a significant reduction of the VGSC-blocking effects induced by phenytoin and lamotrigine in the hippocampus was observed in animals. This observation was more evident in brain tissue with epileptiform activity at higher discharge frequencies (Remy et al. 2003b). Increased VGSC mRNA expression was observed following kainic acid-induced *status epilepticus* in the rat hippocampus. This altered expression could produce hippocampal hyperexcitability (Bartolomei et al. 1997). After pilocarpine-induced *status epilepticus*, the activation window of VGSC in the dentate gyrus increases, a condition that could amplify neuronal depolarization. This alteration is associated with decreased expression of $\beta 2$ (early) and $\beta 1$ (late) subunits (Ellerkmann et al. 2003). The $\beta 1$ subunit modulates the kinetics of VGSC closure (Brackenbury and Isom 2011; Hull and Isom 2018). Significantly, mutations in this subunit dramatically decreased the blocking effect of phenytoin (Lucas et al. 2005) and carbamazepine (Uebachs et al. 2010, 2012) in animal models and cell lines. They have also been associated with generalized epilepsy (Meadows et al. 2002; Wallace et al. 1998) and epileptic encephalopathies (Hull et al. 2020). This evidence suggests time-dependent alterations in the expression of the β -subunit of VGSC that may lead to poor response to ASMs.

7.3 GABA_A Receptors

GABA_A receptors are pentameric proteins composed of different subunits. The endogenous ligand of these receptors is gamma amino butyric acid (GABA), the primary inhibitory neurotransmitter of the central nervous system. When GABA activates GABA_A receptors at postsynaptic sites, phasic inhibition occurs, whereas activation of GABA_A receptors at extrasynaptic sites mediates tonic inhibition (Sigel and Steinmann 2012). Various ASMs, such as benzodiazepines, barbiturates, and topiramate, act on these GABA_A receptors (Greenfield 2013). In hippocampal tissue resected from DRE patients, GABA_A receptors expression is diminished in areas of significant cellular loss. Interestingly, surviving neurons show similar or

increased expression of GABA_A α 1 and α 2 subunits, suggesting a relative increase in receptors per neuron containing these subunits (Ghit et al. 2021; Loup et al. 2000). Since drug-binding sites are located between the α - β and α - γ interfaces, alterations in the expression of these subunits are associated with changes in drug selectivity (Sigel and Steinmann 2012). Positron emission tomography (PET) evaluations revealed focal variations in GABA_A receptor-binding sites in the hippocampus of patients with DRE. A decrease in binding is detected during the days following a seizure (Bouvard et al. 2005), suggesting a diminished therapeutic response to benzodiazepines during the post-ictal and interictal periods. These changes could be associated with cell loss and altered subunit composition of GABA_A receptors, decreasing their sensitivity to ligands (Lamusuo et al. 2000). Interestingly, in vitro analysis of the temporal cortex of patients with DRE revealed increased benzodiazepine binding and increased γ 2 subunit mRNA, which was more evident in patients with lower seizure frequency. These findings lead to suggest that patients with low seizure frequency may present enhanced inhibitory effects mediated by the benzodiazepine binding, an effect not evident in patients with high seizure frequency and drug-resistant phenotype. This study supports that GABA receptor expression and function alterations correlate with clinical factors (Rocha et al. 2015).

Fifty percent of the animals with epilepsy due to pilocarpine-induced *status epilepticus* showed a poor response to phenobarbital. Since hippocampal damage and plasma phenobarbital concentration were similar between responder and nonresponder rats, the lack of response to this ASM could be related to target alterations (Bankstahl et al. 2012). The loss of phenobarbital efficacy in a model of temporal lobe epilepsy model induced by electrical stimulation of the basolateral amygdala was associated with low expression of GABA_A subunits, particularly the α 1 subunit in all the areas analyzed. In contrast, the expression of α 2, α 5, β 2/3, and γ 2 subunits was significantly decreased in the hippocampus. However, these conditions did not correlate with neuronal loss (Bethmann et al. 2008). Consistent with previous evidence, decreased mRNA expression of α 1 and β 3 subunits in the dentate gyrus of rats with epilepsy was associated with a diminished response to benzodiazepine modulation (Brooks-Kayal et al. 1998). These data support that alterations in the expression of the α and β subunits of the GABA_A complex could underlie the loss of efficacy of ASMs that modulate GABAergic neurotransmission in DRE.

7.4 Other Receptors Involved in Drug-Resistant Epilepsy

PET studies revealed that the binding of [¹⁸F]-FCWAY, a ligand with a high affinity for 5-HT_{1A} receptors, is lower in regions close to the epileptic focus (Toczek et al. 2003). In contrast, functional binding assay revealed that the ability to activate 5-HT_{1A} receptor-coupled Gi proteins is more significant in the hippocampus of patients with drug-resistant temporal lobe epilepsy. These observations suggest

overactivation despite the low binding of 5-HT_{1A} receptor-associated transductional mechanisms (Cuellar-Herrera et al. 2014).

DRE is also associated with changes in the opioid system (Burtscher and Schwarzer 2017). Some autoradiographic studies showed that mu-opioid receptor binding is reduced in the neocortical tissue surrounding the epileptic focus of patients with DRE (Ondarza et al. 2002). The neocortex of patients with drug-resistant temporal lobe epilepsy (TLE) shows enhanced mu-opioid receptor binding but low DAMGO-stimulated Gi protein (Rocha et al. 2009). In patients with epilepsy, elevated mu receptor mRNA expression and binding without changes in the DAMGO-stimulated Gi protein are observed in the hippocampus (Cuellar-Herrera et al. 2012). Therefore, these studies support that mu-opioid receptors mediate the changes in expression and transductional mechanisms.

Another system that has gained relevance in recent decades is the endocannabinoid system. At the central level, the endocannabinoid system modulates inhibitory and excitatory neurotransmission through CB1 and CB2 receptors and other noncanonical receptors (Cristino et al. 2020). PET studies in TLE patients revealed that the availability of CB1 receptors in the neocortex ipsilateral to the epileptic focus is increased. This observation is more evident in patients with a shorter latency to the last seizure and a high frequency of ictal events before scanning (Goffin et al. 2011). The efficacy of CB1 receptor-induced G-protein signaling is, indeed, higher in the neocortex and hippocampus in patients with drug-resistant mesial TLE and more evident in patients with no evidence of mood disorders (Rocha et al. 2020). The hippocampal and cortical microvasculature of patients with DRE showed a high efficiency in activating G_{ai/o}-coupled proteins mediated by CB1 and CB2 receptors, a situation that does not correlate with their protein expression (Nuñez-Lumbreras et al. 2021). These findings support the idea that alterations of different receptors or their signaling cascades could explain the drug-resistant phenotype in epilepsy.

7.5 Conditions that Reduce ASMs Effectiveness

7.5.1 Desensitization

Desensitization is the decrease in response after persistent exposure to drug agonist (Caimmi et al. 2019). Therefore, dose increases do not produce the expected effect (Gupta et al. 2018). Desensitization is particularly relevant in epilepsy since it may result from the chronic administration of ASMs (Thijs et al. 2019). It has been reported that in cell lines expressing moderate levels of the human 5-HT_{1A} receptor, stimulation of this receptor with prototypical agonists (5-HT, F13714, befiradol, and NLX-101) reaches a saturation point. In contrast, when this receptor is overexpressed, stimulation with the same agonists increases 5-HT_{1A} receptor activation. However, increasing the concentration of the agonists results in the loss of the intrinsic activity of these receptors until any response occurs

(Newman-Tancredi et al. 2019). The use of full agonists of GABA receptors tends to cause desensitization, which can lead to tolerance and dependence (Janković et al. 2021). Similarly, 2-day exposure to zolpidem, a positive allosteric modulator of GABA_A receptors, induces uncoupling of GABA and benzodiazepine from their binding sites in rat cerebellar granule cells (Vlainić et al. 2012). Moreover, autoradiography and [³H]-muscimol binding experiments demonstrated two agonist-binding sites on GABA_A receptors: a low-affinity one, which requires micromolar concentrations of GABA to be activated, and a high affinity one, where nanomolar concentrations of GABA are sufficient to interact with it; however, the muscimol-functional response decreases, probably as a manifestation of the desensitized form of these receptors (Chandra et al. 2010; Jembrek and Vlainic 2015). The preference for the sensitive or desensitized state is associated with structural changes in the receptor and may be affected by the drug exposure time. In this regard, prolonged exposure to GABA and other GABA agonists such as piperidine-4-sulfonic acid, muscimol, β -alanine, and 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol causes significant desensitization of GABA_A receptors expressed in *Xenopus* oocytes (Akk et al. 2011).

GABA_A receptor desensitization is associated with DR-MTLE patients (Ragozzino et al. 2005). In this regard, GABA current was less efficient in oocytes injected with isolated neocortex membranes from DR-MTLE patients and in neocortical slices from these patients due to repetitive applications of GABA. The desensitization of GABA_A receptors was reduced by levetiracetam exposure in the range of 0.5 to 100 μ M (Palma et al. 2007). Further studies should be carried out to determine the optimal administration of the ASM able to induce therapeutic effects without inducing side effects such as desensitization.

7.5.2 Receptor Downregulation

The availability of receptors at the cell membrane depends on several processes, starting with the expression of the genes that encode them. Expression of these genes leads to protein synthesis of the receptors, which are subsequently transported to the cell membrane and are susceptible to activation after binding to their ligands (Tipton and Russek 2022). In epilepsy, reduced receptor expression compromises the effect of ASMs (Tipton and Russek 2022).

Epileptic activity in preclinical models has been associated with the downregulation of GABA_A receptor subunits and scaffold proteins (González et al. 2013). GABA_A receptor downregulation is also induced following chronic GABA exposure (Roca et al. 1990). Studies have reported that rats subjected to subchronic treatment with diazepam and vigabatrin show lower latency to pentylenetetrazol-induced seizures. This effect is associated with poor binding of the benzodiazepine receptor in several brain areas, indicating a differential decrease of GABA receptors (Rocha 2008). The glutamate transporter GLT-1, a Na⁺-dependent transporter involved in removal glutamate from extracellular space, is downregulated in the brain of patients

with Rasmussen encephalitis (He et al. 2020). Therefore, downregulation of either receptor, ion channels, or transporters associated with the control of excitatory neurotransmission could be actively participating in the expression of the pharmacoresistant phenotype of epilepsy.

7.5.3 Internalization

Internalization of receptors or ion channels that are the pharmacological targets of ASMs results in low efficacy of these drugs and failure to control seizures. Internalization of receptors and ion channels occurs physiologically in cells. However, changes in the microenvironment, such as disease, receptor overactivation, and the presence of drugs, can increase the internalization rate (Estadella et al. 2020). Moreover, GABA_A receptors' internalization occurs acutely and chronically during epileptogenesis (Blair et al. 2004). In addition, GABA_A receptor internalization is associated with losing response to benzodiazepines in convulsive *status epilepticus* in pediatric patients (Singh et al. 2020).

Receptor internalization is a stepwise process. First, as receptors are phosphorylated, they become inactive, limiting their response to agonists. Subsequently, receptors are endocytosed by a clathrin-dependent or clathrin-independent pathway involving other proteins such as RhoA and ARF6 (Estadella et al. 2020; Gong et al. 2008). The receptor endocytosis process takes a few minutes. Once internalized, receptors can remain sequestered for hours (Wang and Marvizón 2002). When receptors are internalized, two processes can occur: the receptors can be recycled and repositioned on the cell membrane or degraded. When the former occurs, the receptors return to the cell surface and, after dephosphorylation, revert to their activatable form. The kinetics of this receptor recycling process is estimated to last between 60 to 250 min (Bhattacharyya et al. 2002; Wang and Marvizón 2002). Therefore, even when ASMs are available in the brain microenvironment, they would not be able to activate the signaling pathways necessary to control seizures. Consequently, this situation may contribute to drug resistance in epilepsy.

7.6 Changes in Receptor Signaling

7.6.1 PIP2 Modifies the Response to ASMs

Different second messengers are involved in transductional mechanisms following ligand binding to the G protein-coupled receptors (GPCR). Second messengers comprise hydrophobic, hydrophilic, and gaseous molecules that transduce extracellular signals into the cell to induce a physiological effect. Hydrophobic second messengers include bioactive lipids such as sphingolipids, arachidonic acid

derivatives, diacylglycerol, and phosphoinositides, which bind to the cell plasma membrane (Newton et al. 2016). Phosphoinositides are acidic phospholipids containing an inositol sugar head and differ from each other depending on the number of phosphate groups on the inositol sugar head (Falkenburger et al. 2010). As such, this family includes phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 3,4,5 trisphosphate (PIP3), among others. Each member of the phosphoinositide family plays a different role in biological processes, from cell growth to ion channel regulation (Suh and Hille 2008).

PIP2 represents the major plasma membrane phosphoinositide interacting with allosteric-binding sites to control the function of several ion channels, such as inwardly rectifying (Kir) and voltage-dependent (Kv) K⁺ channels (Balla 2013). The interaction between PIP2 and Kv7 channels is, in fact, essential for stabilizing the open state of the Kv7 channel and the flow of potassium ions (Suh and Hille 2008; Sun and MacKinnon 2020). In this regard, Kv 7.2 (KCNQ2) and Kv 7.3 (KCNQ3) channels are significant components of M-currents that prevent excessive hyperexcitability by increasing the firing threshold and interspike interval (Lawrence et al. 2006; Soldovieri et al. 2011; Tzingounis and Nicoll 2008; Wang et al. 1998). M-currents could be suppressed by activation of Gq protein-coupled receptors (GqPCRs) through decreased PIP2 levels, as PIP2 is the lipid substrate for phospholipase C (PLC) (Suh and Hille 2008; Zhang et al. 2003) (Fig. 7.1).

M-current suppression is associated with seizures, epileptogenesis, and epileptic syndromes (Ambrosino et al. 2015; Biervert et al. 1998; Greene and Hoshi 2017; Miceli et al. 2015). Furthermore, mutations in Kv7.2 channels affect PIP2 binding inducing abnormal expression and function, which may increase neuronal excitability and underlie KCNQ2-mediated epileptic encephalopathy (Kim et al. 2018). Indeed, Kv7.2 mutation is associated with early-onset epileptic encephalopathy and suppression-burst enhances Kv7/M channel activity (Devaux et al., 2016). Thus, KCNQ2 and KCNQ3 mutations are associated with severe epilepsy syndromes, such as Ohtahara syndrome characterized by drug-resistant seizures, psychomotor retardation, and EEG changes (Soldovieri et al., 2014; Köhling & Wolfart, 2016).

Therefore, lipid metabolism is crucial for Kv channel activity and subsequent modulation of neuronal excitability. In this context, PIP2-dependent interaction between voltage-sensing and pore-forming components has been reported to be essential for the action of retigabine, a KCNQ3 channels activator used to treat diseases characterized by hyperexcitability (Kim et al. 2017). Furthermore, zinc pyrithione (ZnPy), a drug more effective on KCNQ2 than on KCNQ3, undergoes a pharmacological shift when PIP2 levels decrease (Zhou et al. 2013). In other words, PIP2 depletion promotes KCNQ3 to become more sensitive to ZnPy and loses sensitivity to retigabine. Additionally, valproic acid has been shown to exert its antiepileptic effects, at least in part, by preventing M-current suppression and decreasing phosphorylation of Kv7.2 at Ser⁵⁵⁸ through inhibition of AKAP79/150 palmitoylation (Greene et al. 2018; Kay et al. 2015). Valproic acid also reduces pentylenetetrazole-induced seizure activity in cultured rat primary hippocampal

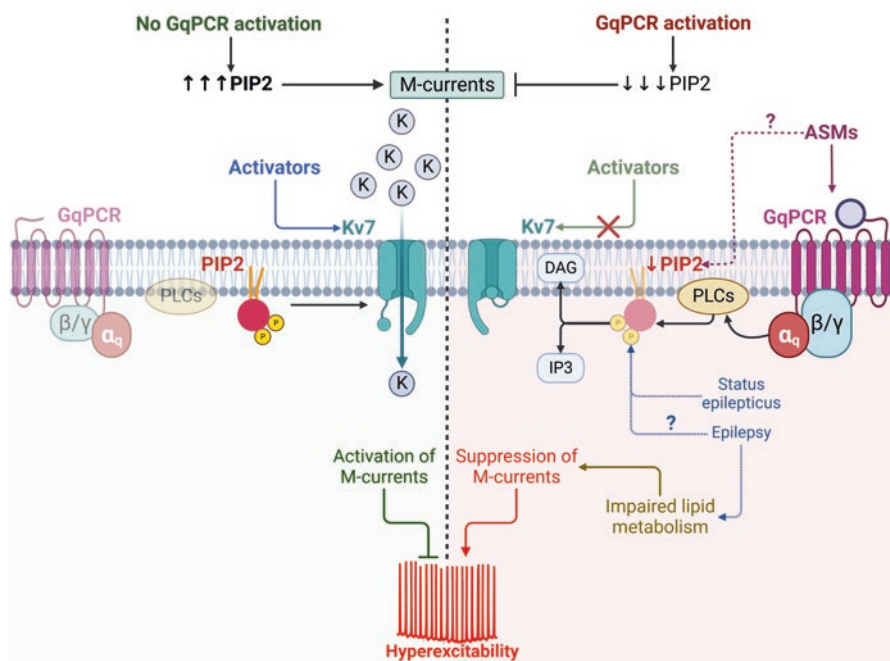


Fig. 7.1 GqPCR modulation of Kv7 channel activity by modifying PIP2 levels. In the absence of Gq protein-coupled receptor (GqPCR) activation (left panel), phosphatidylinositol 4,5-bisphosphate (PIP2) binds to voltage-dependent potassium channels (Kv). The binding of PIP2 to Kv7 channels increases M-currents that prevent neuronal hyperexcitability and regulate the function of Kv7 channel activators. In contrast, binding of an agonist to GqPCR (right panel) triggers phospholipase C (PLC) activity. PLC degrades PIP2 into diacylglycerol (DAG) and inositol trisphosphate (IP3). Increased degradation of PIP2 reduces M-currents and thus promotes hyperexcitability. Furthermore, *status epilepticus* reduces PIP2 levels, and epilepsy impairs lipid metabolism. However, it is still unknown whether epilepsy directly decreases PIP2 levels or whether antiepileptic medications (ASMs) act through the modulation of lipid metabolism. (Figure created using BioRender)

neurons and entorhinal cortex–hippocampal slices by restoring PIP3 and PIP2 levels (Chang et al. 2014).

Finally, it has been proposed that kainic acid induces Ca^{2+} influx in vitro by a TRPV1-related mechanism that depends on the interaction of PIP2 with the receptor (Mohandass et al. 2020). Accordingly, it is possible to suggest that some effects of ASMs depend on PIP2 levels and their proper interaction with ion channels. Therefore, any change in PIP2 metabolism may contribute to the lack of efficacy of some anticonvulsant drugs. In this regard, lipid biosynthesis and metabolism are dysregulated in a Kv1.1 knockout-induced epileptic mouse model (Johnson et al. 2020) and the hippocampus of patients with temporal lobe epilepsy (Ajith et al. 2021); meanwhile, bicuculline-induced *status epilepticus* leads to accelerated degradation of PIP2 (van Rooijen et al. 1986). Based on the above lines of evidence,

it is possible to suggest that the changes in lipid biosynthesis and metabolism observed during epilepsy could be responsible for the decreased functional response to certain drugs. However, further studies are still required to address the relationship between antiseizure medications and levels of bioactive lipids, such as phosphoinositides.

7.6.2 *Lipid Rafts Modify ASMs Effects*

Lipid rafts are cell membrane regions enriched with specific lipids, such as glycolipids, sphingolipids, and cholesterol, which play regulatory roles in synaptic transmission, action potential propagation, and membrane signaling (Levental and Veatch 2016). Within lipid rafts, different constitutive membrane receptors can interact with each other, modulating their signaling (Simons and Toomre 2000). Furthermore, the composition and organization of lipid rafts can modify the signaling pathways activated by the receptors located within them (Levental and Veatch 2016). Therefore, disturbances in lipid raft composition could alter cell functions and the expected response to a drug (Regen 2020). Evidence shows an association between changes in lipid raft composition and the development of different neurological diseases such as Huntington's disease, Alzheimer's disease, Parkinson's disease, depression, and epilepsy (Grassi et al. 2020; Schrattenholz and Soskic 2006).

Lipid rafts are involved in cellular excitability. Electrophysiological and fluorescence analysis in cortical neurons showed that activation of NMDA receptors closer to sodium-calcium exchangers (NCX) within lipid rafts results in main cytosolic calcium entry. Conversely, the destruction of lipid rafts increases the distance between NMDA receptors and NCX and weakens overall cytosolic calcium accumulation (Sibarov et al. 2018). Furthermore, activation of NMDA receptors in cultured hippocampal neurons recruits AMPA receptors to raft domains on the cell surface, increasing the probability of being activated upon glutamate exposure. In contrast, the disruption of lipid rafts reduces the surface expression of AMPA receptors (Hou et al. 2008). The propensity of AMPA receptors to be activated within lipid rafts may reduce the efficacy of ASMs targeting these receptors, as their signaling is biased (Pike 2003).

GABA_{A,B} receptors are also susceptible to entering lipid rafts during the epileptogenic process, whereas inhibiting cholesterol synthesis produces a shift of GABA_{A,B} receptors to non-raft domains (Huo et al. 2009). Translocation of GABA_{A,B} receptors to lipid rafts reduces signal transduction mediated by their activation, affecting the effect of ASMs such as vigabatrin and benzodiazepines (Bouwman et al. 2007; Perescis et al. 2019). Notably, drugs such as pentobarbital reduce NMDA and GABA_A receptors' association with lipid rafts (Sierra-Valdez et al. 2016). Conversely, in vitro experiments revealed that disruption of lipid rafts can potentiate the effect of diazepam on the GABA_A receptor (Nothdurfter et al. 2013).

At present, as the involvement of lipid rafts in drug resistance in epilepsy is unknown, further research is needed.

On the other hand, it is known that it is relevant to indicate that P-glycoprotein, a transporter associated with drug-resistant phenotype, is overexpressed in neurons of patients with pharmacoresistant epilepsy (Lazarowski et al., 2004). When expressed in rafts and non-raft membrane domains, the P-glycoprotein interacts with protein partners regulating the membrane activity and affects trafficking in the cell (Orlowski et al., 2006). Further studies are necessary to elucidate the role of P-glycoprotein overexpression in lipid rafts of neurons of patients with drug-resistant epilepsy.

7.6.3 Oligomer Receptor Complexes in Drug-Resistant Epilepsy

Protein oligomerization is an association between two or more protein subunits to form a high-order complex (Ward et al. 2013). Protein aggregates are composed of the same protein subunit (homo-oligomers) or different protein subunits (hetero-oligomers) (Ali and Imperiali 2005). Since the formation of homo- and hetero-oligomeric complexes modifies the response to drugs in various neuropathologies, it has become a new topic of study (Fuxe et al. 2014). Several studies have shown that receptor oligomerization affects pharmacological and cell signaling responses (González-Maeso 2011). Under various conditions, the physiological role of oligomeric receptors in learning processes and cognitive functions, among others (Borrito-Escuela et al. 2017), could become pathological and lead to disease (Pérez de la Mora et al. 2022).

The formation of oligomeric complexes modifies not only the structure of the receptors but also their functionality (Ginés et al. 2000). The functional implication of receptor oligomeric complexes lies simultaneously in the signaling changes after activating their elements. Evidence has shown that dimer formation modifies the normal signaling of its component receptors (Parmentier 2015). In this regard, it is known that activation of the cannabinoid receptor CB1 leads to the phosphorylation of Gi proteins, inhibiting cyclic adenosine monophosphate (cAMP) synthesis. Similarly, the dopaminergic receptor D2 also activates the Gi protein pathway, with a consequent decrease in cAMP in the cell. Interestingly, the CB1 receptor and the D2 receptor can form a heterodimeric CB1/D2 complex that stabilizes the active state of the CB1 receptor with increased Gs-protein coupling (Fig. 7.2). This stimulates the CB1/D2 complex and increases cAMP concentration. In other words, the effect of CB1/D2 complex activation is opposite to that produced by the activation of the CB1 and D2 receptors separately (Hudson et al. 2010).

Moreover, dimer formation can also modify signaling by recruiting proteins such as β -arrestin. Heterodimerization of μ and δ opioid receptors leads to constitutive recruitment of β -arrestin2. Consequently, downstream signaling pathways, such as

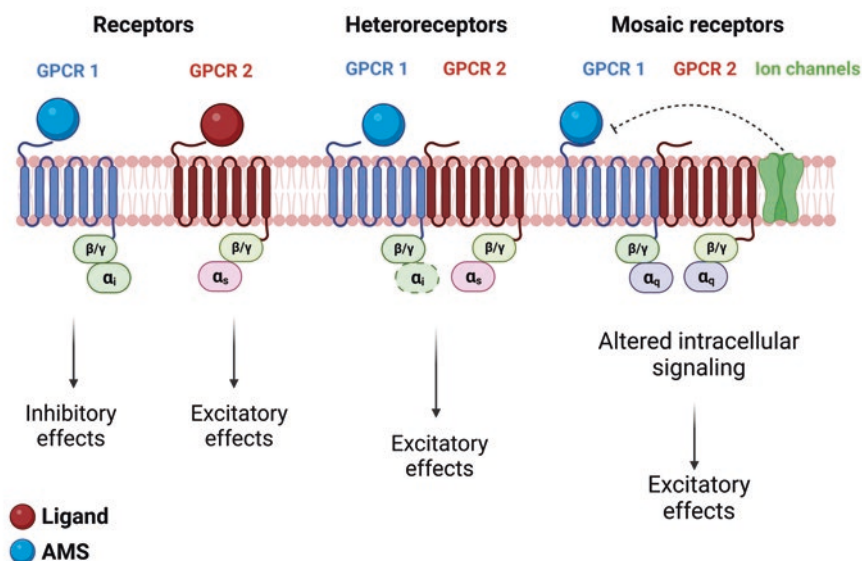


Fig. 7.2 Heteroreceptor and mosaic receptor formation may contribute to drug-resistant epilepsy. Pharmacological targets, such as G-protein-coupled receptors (GPCR) or ion channels, can associate to form heteroreceptors. Heteroreceptors change the intracellular signaling of antiseizure medications (ASM). These signaling changes involve silencing inhibitory pathways and shifting activation routes that can produce excitatory effects and, consequently, could favor the drug-resistant phenotype of patients with epilepsy. (Figure created using BioRender)

ERK1/2 phosphorylation patterns, are altered compared to those observed with individual μ and δ receptors (Rozenfeld and Devi 2007).

Several molecular studies have shown that Na^+ channel α -subunits can interact as dimers (Clatot et al. 2017). Oligomerization between mutant and wild-type VGSC α -subunits leads to an impaired gating probability (Clatot et al. 2018). The β_3 subunit of the VGSC heterooligomer reduces the effect of carbamazepine (Sokolov et al. 2018). Heterodimerization of the Q_2 and Q_3 subunits of voltage-gated potassium channels increases the susceptibility of the channel to retigabine (Li et al. 2020). In addition, mutations in the GABA_A receptor complex increase the probability of this receptor oligomerization and facilitate seizure activity (Wang et al. 2016). Homoreceptor and heteroreceptor complexes regulate each other, and their imbalance is associated with neurological disorders (Borrito-Escuela et al. 2017). In DRE, the lack of efficacy of ASMs may be related to dynamic oligomer formation (Borrito-Escuela and Fuxe 2019).

Another type of oligomeric complexes is mosaics, which refers to the physical receptor–receptor association within the intermembrane space (Agnati et al. 1980). These mosaics can be composed of homo- or heteroreceptors (Agnati et al. 2005a;

Fuxe et al. 2008, 2009). Some examples of heteroreceptors have been established between κ - and δ -opioid receptors, adenosine A1 and A2A with dopaminergic D1 and D2 receptors, respectively (Borrito-Escuela and Fuxe 2019; Ginés et al. 2000).

Activation of mosaic receptors alters the function of other receptors (horizontal network) or intracellular transduction signals (vertical network) of different receptors (Agnati et al. 1980, 2005b). In addition, heterodimerization of adenosine A1 and A2A receptors allows adenosine to exert a fine-tuning modulation of striatal glutamatergic neurotransmission, providing a switching mechanism whereby low and high concentrations of adenosine inhibit and stimulate, respectively, glutamate release (Ciruela et al. 2006).

Based on NMDA-D2 heteroreceptor expression in the striatum, D2 receptor activation can convert glutamatergic-induced long-term potentiation into long-term depression (Higley and Sabatini 2010). In Parkinson's disease, alterations in synaptic signaling are associated with the expression of dopaminergic receptor heteroreceptors with adenosine, neurotensin, or different dopamine receptor subtypes (Borrito-Escuela et al. 2017). Different heteroreceptors (serotonergic and metabotropic glutamate receptors, neurotensin and dopamine receptors, and adenosine and dopamine receptors) are associated with the pathophysiology of psychosis and schizophrenia (González-Maeso et al. 2008; Tanganelli et al. 2012). Dopamine D2-adenosine A2A heteroreceptors lead to acute locomotor changes and cocaine-induced sensitization (Filip et al. 2006). In addition, cocaine-induced psychostimulation is associated with the formation of heteroreceptor complexes between dopamine D2 receptors and NR2B subunits of the NMDA receptor in the neostriatum (Liu et al. 2006). In patients with different neuropathologies (e.g., depression), activation of fibroblast growth factor 1 and 5-hydroxytryptamine 1A heteroreceptor complexes could lead to the neuroprotective effect (Borrito-Escuela et al. 2012).

Patients with DRE are characterized by nonresponsiveness to medication, even when it is directed to more than one pharmacological target (Kwan et al. 2010). The expression of homo- and hetero-oligomer complexes and mosaic receptors with consequent changes in the effects of specific targets may explain the resistance to multiple ASMs with different mechanisms of action in patients with DRE. If this is the case, it is essential to establish new pharmacological strategies based on homo- and heterooligomer complexes rearrangement in synaptic and extra-synaptic regions in individuals with epilepsy (Borrito-Escuela and Fuxe 2019).

Proteins regulate cellular processes by forming homo- or hetero-oligomerizations in the cellular environment. Dysregulation of these processes may lead to the expression of the pharmacoresistant phenotype in epilepsy (Singh and Jois 2018). Future studies should determine whether the DRE condition is associated with the dynamic expression of mosaic receptors.

7.7 Epigenetic Changes in ASM Targets

DRE is characterized by the failure of adequate trials of two or more ASMs (alone or in combination) to achieve sustained seizure freedom (Kwan et al. 2010). The loss of efficacy of ASMs with distinct mechanisms of action indicates simultaneous changes in several targets. In this section, we present information supporting epigenetic changes to explain different mechanisms of target resistance.

Epigenetics has been defined as “changes in gene function that are heritable and do not depend on changes in the DNA sequence” (Deans and Maggert 2015; Holliday 1994). The nucleosome core comprises 147 bp of DNA wrapping 1.65 times the histone octamer and includes 14 contact points between histones and DNA. This structure facilitates packaging function and ensures stability under physiological conditions. However, it is essential to note that it is highly dynamic and that several protein complexes interact to regulate its configuration. Histone tails and globular domains are subject to several posttranslational modifications; one of the most studied is the acetylation of histones 3 and 4, which is associated with active transcription. Histone acetylation is performed by histone acetyltransferases, which acetylate lysine residues and change the net charge of nucleosomes, thereby loosening DNA-histone interactions. Notably, the biological function of gene expression is determined by the number of modified lysine residues and not by specific acetylated regions (Egger et al. 2004; Li et al. 2007).

Histone deacetylases (HDAC 1-11) are the enzymes responsible for the deacetylation of lysine residues and, thus, promote transcriptional repression. Increased expression of class I HDAC has been described in the resected temporal cortex of patients with DRE. Class I HDAC is notably expressed in neurons but not glia (Huang et al. 2012). Its overexpression has been associated with impaired memory formation and spatial learning, decreased dendritic arborization, diminished synaptic plasticity, and suppression of the expression of genes that regulate neuronal activity and synaptic remodeling (Guan et al. 2009).

In animal models of epilepsy and human tissue from patients with DRE, increased HDAC2 expression has been observed in the temporal neocortex (Huang et al. 2012). HDAC2 downmodulates the face of the benzodiazepine-sensitive GABA_A α 1 subunit (Enna 2007; Zhang et al. 2019) and the GluA2 subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor (Huang et al. 2002). Notably, the GluA2 subunit is responsible for calcium impermeability and modulates trafficking and tetramerization of the AMPA receptor (Greger et al. 2003; Wright and Vissel 2012). GluA2 subunit mutations are associated with several neurodevelopmental abnormalities and epileptic encephalopathies (Salpietro et al. 2019). This evidence suggests that epilepsy causes deacetylation of the GluA2 promoter, which decreases GluA2 subunit expression, leading to calcium-permeable AMPA receptors and, thus, enhancing glutamate neurotransmission (Pellegrini-Giampietro et al. 1997).

DNA methylation is a dynamic and reversible epigenetic change that induces gene silencing (Egger et al. 2004). DNA methyltransferases (DNMT) are a family

of highly conserved enzymes that catalyze the addition of methyl groups to the CpG islands of promoter regions present in genomic DNA (Lyko 2018). Overexpression of DNMT1 and DNMT3 was reported in resected neocortical tissue from DRE patients (Zhu et al. 2012). DNMT1 conserved DNA methylation, whereas DNMT3 induced de novo DNA methylation (Lyko 2018). These results were consistent with those observed in autopsy brain samples from schizophrenia patients in which both DNMT1 and DNMT3 mRNAs were upregulated in GABAergic neurons (Zhubi et al. 2009).

Genome-wide methylation analysis of resected hippocampal tissue from DRE patients revealed 119 hypermethylated and 27 hypomethylated genes compared to autopsy samples. Gene ontology of the altered genes revealed alterations in developmental processes, cell development, differentiation, and death. Hypermethylated genes expressed in neurons in DRE patients included calcium, potassium, and sodium channels, proteins associated with neuronal maturation, and proteins related to synaptic transmission (Miller-Delaney et al. 2015).

Noncoding RNA transcripts do not encode proteins but modulate transcription by targeting transcriptional activators or repressors through regulating chromatin structure. Among the noncoding RNA, microRNAs (miRNAs) are typically between 21-28 nucleotides in length and act by targeting homologous sequences in the genome (Chen and Rajewsky 2007; Goodrich and Kugel 2006). Individual miRNAs are fine-tuning gene modulators as they can interfere with the expression of hundreds of proteins, although their effects are generally modest (Brennan and Henshall 2020).

In hippocampal tissue resected from patients with DRE, Dicer protein expression was reduced in samples with sclerosis but unchanged in those with moderate hippocampal pathology. Dicer is a protein that matures miRNAs, and its downregulation in DRE patients was associated with lower expression of several miRNAs, including miRNA-92a (McKiernan et al. 2012). Interestingly, miRNA-92a can downregulate AMPA receptor expression (Letellier et al. 2014). In epilepsy, downregulation of Dicer expression would induce a decrease in miRNA-92a and, thus, an increase in AMPA receptor expression, which could lead to hyperexcitability.

Overexpression of miRNA-134 has also been found in hippocampal tissue resected from DRE patients (Jimenez-Mateos et al. 2012; Reschke et al. 2017). These data suggest that miRNA-134 overexpression would impair synaptic plasticity. Overexpression of miRNA-134 was detected in hippocampal tissue from mice previously submitted to *status epilepticus* (Jimenez-Mateos et al. 2012, 2015; Reschke et al. 2017). Interestingly, miRNA-134 knockdown had an antiepileptogenic effect associated with decreased neuronal death and astrogliosis (Jimenez-Mateos et al. 2012). Similarly, miRNA-134 knockdown produced antiepileptogenic effects in rats subjected to electrical stimulation of the perforant pathway (Reschke et al. 2017). These data reveal that miRNA-134 is associated with the pathogenesis of seizures and epilepsy and is involved in brain excitability.

Furthermore, miRNA-155 overexpression in resected hippocampal tissue from DRE patients correlated with increased seizure frequency, sclerosis, and reduced probability of post-surgery seizure freedom (Huang et al. 2018). A similar increase of miRNA-155 was found in the hippocampus of rats with epilepsy (Huang et al.

2018). Notably, miRNA-155 was overexpressed 2.5-fold in the cerebrospinal fluid of patients whose epilepsy surgery did not modify the occurrence of seizures. Bioinformatic analysis revealed that miRNA-155 inhibits *SCN1A* gene expression (Zhang et al. 2018). The *SCN1A* gene encodes the pore-forming α -subunit of VGSC NAV1.1. The α -subunit forms the ion-conducting pore and contains voltage sensors that dictate part of its kinetics. This channel's mutation has been associated with reduced excitability of GABAergic neurons, which preferentially express Nav1.1 (Catterall et al. 2010; Ragsdale 2008). This evidence suggests that epilepsy-induced increased expression of miRNA-155 may result in low SCN1A expression, thus reducing GABAergic neurotransmission.

7.8 Conclusions

Changes in ASMs targets involve structure, expression, oligomer formation, and membrane localization modifications. In addition, changes at the intracellular signaling level may also be involved, reducing the effects of ASMs, or even changing their pharmacological effects and facilitating neuronal excitability. Both structural and signaling alterations could explain why some patients develop the drug-resistant phenotype in epilepsy. It is essential to consider these alterations and search for new therapeutic strategies to control the pharmacoresistant condition in epilepsy.

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Chapter 8

Cellular and Molecular Mechanisms of Neuroinflammation in Drug-Resistant Epilepsy



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Abstract Drug-resistant epilepsy (DRE) is characterized by the persistence of epileptic seizures despite the administration of antiepileptic treatments. The pathogenesis of this condition has been associated with many different molecular and cellular mechanisms. In this chapter, we describe how the CNS immune response is associated with the onset of epilepsy and the molecular mechanisms underlying brain injury associated with neuroinflammatory response triggered by epileptic seizures. Next, we describe the possible role of neuroinflammation in the progression from epilepsy to DRE.

Keywords Drug-resistant epilepsy · Innate immune system · Adaptive immune system · Blood-brain barrier · Oxidative stress

8.1 Introduction

Epilepsy is a progressive neurological disease characterized by unprovoked seizures, namely, bursts of electrical neuronal activity resulting from hypersynchronization neuronal discharges (Devinsky 2022; Devinsky et al. 2018). This disease

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affects approximately 70 million people worldwide (Beghi et al. 2015; Thurman et al. 2011).

In some patients, epilepsy can manifest as a pharmacologically intractable form. The International League Against Epilepsy (ILAE) defines this drug-resistant epilepsy (DRE) as persistent epileptic seizures despite the administration of an antiepileptic treatment (monotherapy or combining two or three antiepileptics) (Kwan et al. 2010; Scheffer et al. 2017). The most prevalent DRE is mesial temporal lobe epilepsy (mTLE), in which approximately 30% of patients become refractory to treatment (Denton et al. 2021; Kalilani et al. 2018; Loscher and Brandt 2010). DRE is associated with several factors, including the type of epilepsy, the severity of the seizures, the extent of the brain damage, the patient's age, genetic background, and epigenetic factors (Crossley et al. 2013; Vezzani et al. 2019).

One of the most accepted hypotheses to explain DRE involves neuroinflammation in the pathogenesis of this condition (Shimada et al. 2014; Vezzani 2004). It has been reported that damage to the blood-brain barrier (BBB) and/or the activation of non-neuronal cells such as astrocytes and microglia promote the synthesis and release of cytokines and chemokines, generating neuronal hyperexcitability, which increases the severity of the seizure activity and favors the development of recurrent seizures (Dupuis and Auvin 2015; Paudel et al. 2018; Ravizza et al. 2018). In addition, autoantibodies have been detected in the serum and cerebrospinal fluid (CSF) of patients with DRE (Alyu and Dikmen 2017; van Gassen et al. 2008; Vezzani et al. 2019), suggesting that both, the innate and the adaptive immune systems are involved in the development of DRE. Moreover, this hypothesis has gained interest because some steroids and anti-inflammatory drugs show neuroprotective effects and help to reduce the frequency and severity of seizures without anti-epileptogenic actions; however, the mechanisms underlying their effects are still unknown (Dey et al. 2016).

In this chapter, we discuss the role of the CNS immune response in the onset of epilepsy and analyze some molecular mechanisms involved in the neuroinflammatory response triggered by epileptic seizures, which could be associated with the development of DRE.

8.1.1 The Immune Response in the CNS

The immune system enables a living organism to discriminate between “self” and “nonself” to prevent or limit the development of infections. The immune system has two arms: the innate and the adaptive immune systems (Iwasaki and Medzhitov 2010; Vezzani et al. 2019).

The innate immune system is the first defense against invading pathogens or foreign objects; it mounts an immediate, non-specialized response to prevent germ invasion (Iwasaki and Medzhitov 2015). This first line of defense includes anatomic barriers, such as the skin and mucous membranes, physiological changes aimed at preventing pathogen life cycles, such as fever and low pH, and the activation of

innate immune cells like phagocytes, neutrophils, macrophages, natural killer cells (NK cells), mast cells, basophils, dendritic cells, and eosinophils (Iwasaki and Medzhitov 2015). The cells of the innate immune system can immediately mount an unspecific response against an immune challenge and retain no immunological memory (Iwasaki and Medzhitov 2015; Kumar et al. 2011). These cells become activated through the binding of pathogen-associated molecular patterns (PAMPs) to their pattern-recognition receptors (PPRs) (Barra et al. 2010; Bert et al. 2022; Varghese et al. 2022).

In contrast, the adaptive immune system is highly specific and creates antigen-specific T and B lymphocyte clones that express specific antigen receptors to generate immunological memory (Iwasaki and Medzhitov 2015; Varghese et al. 2022). B lymphocytes drive the response to harmful substances in body fluids like the blood and CSF; they constitute the humoral immune response, characterized by the production of antibodies (Holodick et al. 2014). This humoral response is triggered by macromolecules like: secreted antibodies, complement proteins, and antimicrobial peptides, resulting in antibody production (Garraud and Li 2015).

On the other hand, cell-mediated immunity does not depend on antibodies and entails a specific cell response to a pathogen that activates phagocytosis, antigen-specific cytotoxic T lymphocytes, and cytokine release. T lymphocytes recognize and attack pathogens directly or indirectly by interacting with antigen-presenting cells through PRR activation. This activation results in cytokine secretion and the upregulation of co-stimulatory molecules necessary to induce T cell responses (Ribot et al. 2021; Velardi et al. 2021). Furthermore, T cells also express PRRs, suggesting that they can be directly activated by PAMP interaction (Palm and Medzhitov 2009).

In addition, the secretion of proinflammatory mediators regulates the physiology of the diverse organs, including the brain (Dinarello 2000), since these signals reach the CNS. The brain detects proinflammatory cues through two different routes: a neural pathway and a humoral pathway. The neural pathway implies the expression of cytokine receptors in nerve terminals such as those of the vagus nerve. Meanwhile, the humoral pathway involves transporting proinflammatory factors through the blood to the CNS, either by reaching the circumventricular organs or altering blood-brain barrier (BBB) permeability (Xie et al. 2021). Furthermore, under pathological conditions, inflammation facilitates the access of inflammatory cytokines and immune cells into the BBB capillaries, altering the physiology of the brain parenchyma (Banks and Erickson 2010; Shichita et al. 2012).

The CNS was previously considered an immune-privileged site due to the BBB (Pachter et al. 2003). However, at least two new findings contradict this concept: the presence of lymphatics in the meninges (Da Mesquita et al. 2018) and the harboring of several resident cells with immune capacities (Rivest 2009). These findings indicate that the brain is far from not mounting an immune response.

On the other hand, endothelial cells from the blood vessels of the BBB are partially covered by pericytes, which regulate BBB permeability by modifying capillary diameter. Moreover, astrocytes that are associated with endothelial cells in the BBB are also involved in the immune response of the CNS by secreting

proinflammatory mediators that increase BBB permeability (Erickson and Banks 2018; Erickson et al. 2020; Pandey et al. 2016).

Microglia are the resident macrophages of the brain. These cells were found surrounding dying Purkinje cells in the developing cerebellum, highlighting their role in the phagocytosis of neural progenitors. Microglia-mediated neuronal clearance was also observed in the developing cortex and the subventricular zone (SVZ) of the adult mouse brain. Microglia also engulf neural progenitors during neuronal damage; this process occurs through the activation of P2RY12 receptors by ATP. Furthermore, microglia participate in the clearance of neuronal material; in the eye, the engulfment of retinogeniculate neuronal material depends on the complement system (Cq1, C3, and CR3), but in the hippocampus, it involves the TREM2 receptor (Norris and Kipnis 2019).

Microglial activation is a repairing mechanism during a brain insult, but its persistent activation can induce neuronal damage. Microglia can be activated into two phenotypes, M1 and M2. The M1 phenotype is triggered by harmful stimuli such as trauma, damage-associated molecular patterns (DAMPs), misfolding proteins, and tumors. M1 microglia has proinflammatory effects as it releases the cytokines TNF α , IL-1 β , and IL-6 that induce neuronal death, myelin impairment, oligodendrocyte necrosis, and BBB damage. Conversely, the M2 phenotype is triggered by anti-inflammatory mediators such as IL-10, IL-4, and TGF β . M2 microglia release anti-inflammatory molecules such as IL-10 and have an array of other functions: they increase the clearance of misfolded proteins, induce the expression of myelin basic protein and myelin oligodendrocyte protein (which support myelination), maintain BBB integrity by promoting vascular renewal and remodeling, and have increased phagocytic activity of damaged neurons and cell fragments (Chai et al. 2022).

Other cells from the peripheral immune system are also involved in the immune response in the brain, either from the periphery or the skull bone marrow. Macrophages and T cells are recruited after CNS injury, and their absence in the brain increases the progression of neurodegenerative diseases in immunosuppressed mice models (Kwon 2022). Bone marrow-derived mast cells recruited in CNS reside in the meninges, choroid plexus, perivascular space, and some brain regions (Erickson and Banks 2018).

Under physiological conditions, a few leukocytes can infiltrate into the perivascular space of the BBB. However, during neuroinflammation, cell infiltration can reach the brain parenchyma. Healthy brains contain considerable numbers of perivascular macrophages and dendritic cells and minor proportions of T, B, and NK cells. CD4 T cells have a role in neurodegenerative diseases in humans and mice; however, the CD69+ CD4 T cell population was observed near microglia in healthy mouse and human brains. Importantly, T CD4 cell-deficient mice displayed a decreased expression of the transcription factors AP-1, Klf4, and Erg1, involved in microglia maturation. The mature microglia markers Fos and Jun were also modified, affecting synaptic pruning in these mice (Pasciuto et al. 2020).

In physiological conditions, monocytes derived from the skull and vertebrae bone marrow can migrate to the dural meninges, where they differentiate into

resident macrophages. The passage of monocytes from the bone marrow to the dura mater is mediated by channels that connect both areas, creating an anatomical pathway for myeloid cell migration. These cells can infiltrate CNS parenchyma during injuries, such as experimental autoimmune encephalomyelitis, spinal cord injury, or optic nerve damage. Their phenotype is more regulatory than inflammatory, suggesting that they attenuate the immune response in the brain (Cugurra et al. 2021).

8.1.2 The Blood-Brain Barrier and the Inflammatory Response During Epilepsy

The BBB is a highly selective interface between blood and the CNS parenchyma. The BBB maintains an appropriate parenchymal microenvironment that prevents the diffusion of toxic substances into the brain; hence, it exerts tight control over the solutes accessing the CNS (Alajangi et al. 2022).

BBB consists of endothelial cells held together by tight junctions surrounded by pericytes, astrocytes, neurons, and microglia; these cells form the neurovascular unit (NVU). The BBB also includes a basement membrane secreted by all the cells forming the NVU (Banks 2015).

Although, strictly speaking, endothelial cells are not considered part of the immune system, their integrity is important to prevent the access of dangerous agents into the brain. Endothelial cells form tight junctions that seal the space between cells, known as the paracellular route. In addition, they also present adherent junctions that stabilize the interactions between cells. These endothelial junctions make brain blood vessels practically impenetrable to polar solutes. Endothelial cells maintain NVU integrity through TGF β and PDGF β secretion, which promote the differentiation and polarization of astrocytes and pericytes (Erickson and Banks 2018).

Endothelial cells allow the selective transport of substances between the blood and the brain parenchyma, which can occur through paracellular and transcellular pathways. Low-mass molecules diffuse passively through paracellular mechanisms regulated by tight and adherent junctions and by the transcellular pathway crossing the phospholipids of the endothelial cell membrane. Nutrients such as glucose, amino acids, small peptides, and organic molecules can be transported transcellular across the BBB. This transport is mediated by specialized solute transporters (SLCs) located in both the luminal and parenchymal sides of the endothelial cells with a polarized distribution. Conversely, the efflux of waste substances and xenobiotics is carried out by pumps such as P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) (Erickson and Banks 2018; Erickson et al. 2020; Pandey et al. 2016).

Pericytes partially cover endothelial cells and are located between astrocytes and the basement membrane. They regulate BBB permeability and blood flow because they can modify the diameter of brain vessels through their contractile properties. Furthermore, pericytes synthesize PDGF, ANG1, TGF β , and sphingosine

1-phosphate, all necessary for the maintenance and survival of endothelial cells (Erickson et al. 2020).

Likewise, astrocytes are located in the brain parenchyma near the basement membrane. They communicate with endothelial cells through endfeet projections; this interaction is crucial for tight junction integrity and, thus, BBB permeability. Furthermore, astrocytes contribute to BBB formation during development, act as glucose storage to provide the energy requirements to neurons, and participate in the clearance of waste and metabolites. During inflammation, astrocytes secrete proinflammatory cytokines, chemokines, and VEGF, which downregulate tight junctions, increase permeability, and promote angiogenesis (Erickson and Banks 2018; Erickson et al. 2020; Pandey et al. 2016).

Brain cells also regulate blood flow. Neurons promote neurovascular density and regulate the blood supply by releasing glutamate to modulate vasodilatation (Hawkins 2009). Microglia contribute to angiogenesis during development and modulate micro-vasculature contractibility during adulthood, regulating BBB integrity. After a brain insult, microglia migrate to the injured site and phagocytose harmful substances (Erickson and Banks 2018). Furthermore, sustained inflammation promotes phagocytosis of astrocytic endfeet and leukocyte recruitment by microglia (Knox et al. 2022).

In 1986, Cornford was the first to report that the BBB responds to seizures (Cornford and Oldendorf 1986). Later, the postmortem brains of epileptic patients were shown to present an increase in pinocytotic markers, tight junction alterations, and thickening of the basement membrane of BBB. Moreover, the epileptic focus had lower expression of glucose transporter-1 and, consequently, less brain glucose uptake and hypometabolism (Cornford 1999; Janigro 1999). Since then, it has become clear that epilepsy alters the function of the different cell types in the BBB.

In both epileptic patients and animal models of epilepsy, activation of the microglia promotes inflammation, clearance of neurons and debris, and changes in neuronal activity that promote chronic seizures (Bosco et al. 2020). Drug-resistant patients with temporal lobe epilepsy (TLE) also display proinflammatory activity (Liu et al. 2022). This type of epilepsy triggers the brain's innate immune response mediated by microglia and astrocytes, which depend on Toll-like receptors 2 and 4 (TLR2 and TLR4), respectively. In patients with TLE, TLR-mediated microglial activation induces the secretion of proinflammatory cytokines like IL-1 β , CXCL8, IL-6, and TNF α after a seizure. Moreover, microglia impair GABAergic and glutamatergic neurotransmission, leading to increased neuronal excitability (Almeida et al. 2022). Astrocytes usually modulate neurotransmitter homeostasis, but in neuroinflammatory conditions, astrocytes increase excitatory synapses and seizure susceptibility. In mTLE, hippocampal astrocytes reduce their activity and expression of glutamine synthetase, impairing glutamate conversion and increasing neuronal excitation (Almeida et al. 2022; Chen et al. 2022).

Blood-borne monocytes infiltrate the brain, contributing to the onset of seizures (Bosco et al. 2020). Infiltrated monocytes can be distinguished from microglia due to their short life, round soma, limited processes, and the expression of CCR2. However, monocytes differentiate into macrophages in pathological conditions like

seizures, making it difficult to distinguish them from activated microglia (Bosco et al. 2020).

Monocyte recruitment after seizures was proposed to be mediated by CCL2 since this chemokine is upregulated in epileptic patients and secreted by microglia, astrocytes, endothelial cells, and damaged neurons. CCL2 interacts with CCR2 to increase BBB permeability and facilitate monocyte infiltration. Furthermore, CCL2-CCR2 signaling during neuroinflammation and seizures could induce monocyte differentiation into IL-1 β -secreting macrophages (Bosco et al. 2020).

Neutrophils are also recruited after seizures; CD4+ and CD8+, Treg, and Th17 cells can infiltrate the brain parenchyma and produce IL-17, contributing to DRE in pediatric patients. ICAM-1 and ICAM-2 adhesion molecules also participate in Th1 and Th17 cell migration through the BBB. Furthermore, inflammatory mediators secreted from Th cells, such as IL-1 α , IL-1 β , CCL1, CCL3, CCL4, and CCL5, have also been observed in the brain of patients with TLE (Liu et al. 2022).

Albumin infiltration in brain parenchyma has evidenced BBB damage in patients with DRE. BBB disruption can result from epilepsy since BBB permeability increases from hours to days after status epilepticus. Moreover, other conditions that lead to BBB impairment, such as inflammation, infection, and stroke, can induce seizures. Furthermore, mannitol-induced BBB opening increases the frequency of seizures in epileptic rats (Archie et al. 2021). Increased expression of astrocytic VEGFR-3 was observed in patients with TLE and animals with pilocarpine-induced status epilepticus. Additionally, high proliferation of endothelial cells and morphological changes in pericytes have also been found in acute seizure models (Chen et al. 2022). Importantly, P-gp, which plays a key role in drug efflux, is overexpressed in neuroinflammation induced by multiple seizures. The overexpression of P-gp has been widely associated with resistance to antiepileptic drugs (Erickson and Banks 2018).

8.2 Molecular Mechanisms Related to Inflammation in Drug-Resistant Epilepsy

As mentioned previously, inflammation is an essential response to infection, aging, trauma, or damage involving cells within the CNS and proinflammatory molecules and neurological diseases such as epilepsy (Soltani Khaboushan et al. 2022). Furthermore, systemic inflammation may disrupt the BBB and allow the infiltration of immune cells into the brain parenchyma, generating neuroinflammation (Bendorius et al. 2018; Mukhara et al. 2020).

The neuroinflammatory response is classified into two stages: acute and chronic. The acute stage is associated with phagocytosis, glial cell activation, the release of reactive oxygen and nitrogen species (ROS and NOS), cytokines, chemokines, and the recruitment of peripheral blood cells in the brain. While the chronic stage is a prolonged and persistent inflammatory response that fails to resolve, with sustained

release of inflammatory mediators, increased oxidative stress, activation of microglia, and cell damage that induce neurodegeneration (Campos-Bedolla et al. 2022; Riazi et al. 2010).

A successful neuroinflammatory process should eliminate a pathogen and/or initiate tissue damage repair. However, various studies have reported that the failure to modulate the neuro-inflammatory response appropriately can be a determining factor for neurodegeneration, epileptogenesis, and DRE (Vezzani and Friedman, 2011; Barker-Haliski et al. 2017; Khansari and Sperlagh 2012; Shabab et al. 2017). In the next sections, we address the neuroinflammatory mechanisms involved in DRE.

8.2.1 *Toll-Like Receptors*

In recent years, Toll-like receptors (TLRs) have been described as participating in the generation of seizures and hippocampal tissue from TLE patients. TLR4 and its ligand, the DNA-binding protein, and the high mobility group 1 box (HMGB1), which acts as a damage-associated molecular pattern (DAMP) under pathological conditions, have a key role in epilepsy (Zhang et al. 2022).

HMGB1 is released by necrotic cells and can either be retained in the nuclei of apoptotic cells or actively released from the nucleus during pyroptosis and inflammasome activation. Extranuclear HMGB1 can be partially oxidized by forming a disulfide bond between C23 and C45; this reaction generates the disulfide isoform of HMGB1, which act as a TLR4 activator that mediates pro-inflammation (Lu et al. 2014a). Conversely, intracellular HMGB1 is fully reduced (all thiol state) and has chemoattractant properties when released extracellularly (Lu et al. 2014a, b; Zhao et al. 2017).

HMGB1 is actively released by neurons and glia after inflammasome activation (Fig. 8.1b). It subsequently activates the PRRs TLR4 and Receptor for Advanced Glycation End (RAGE) in target cells. HMGB1 participates in neurodevelopment, contributing to neuritic growth and cell migration. However, in the adult brain, it has been related to the generation and amplification of the neuroinflammatory response (Lu et al. 2014a, b).

In experimental models of epilepsy, HMGB1 translocates from the nucleus to the cytoplasm of neurons and glial cells at the lesion site, favoring its extracellular release (Maroso et al. 2010). Maroso et al. (2010) reported that HMGB1 translocates to the neuronal cytoplasm one hour after seizure induction in models of acute convulsive seizures induced by chemoconvulsants or electrical kindling. In addition, HMGB1 expression persisted after seizure activity (Maroso et al. 2010). These data indicate that HMGB1 translocation and subsequent release depend on epileptic neuronal hyperexcitability. Similarly, recurrent seizure activity triggers the upregulation of TLR4 and RAGE, a multiligand receptor that binds to advanced glycation end products (AGEs) in neurons, astrocytes, and cerebral micro-vessels (Iori et al. 2013; Maroso et al. 2010; Maroso et al. 2011; Zurolo et al. 2011).

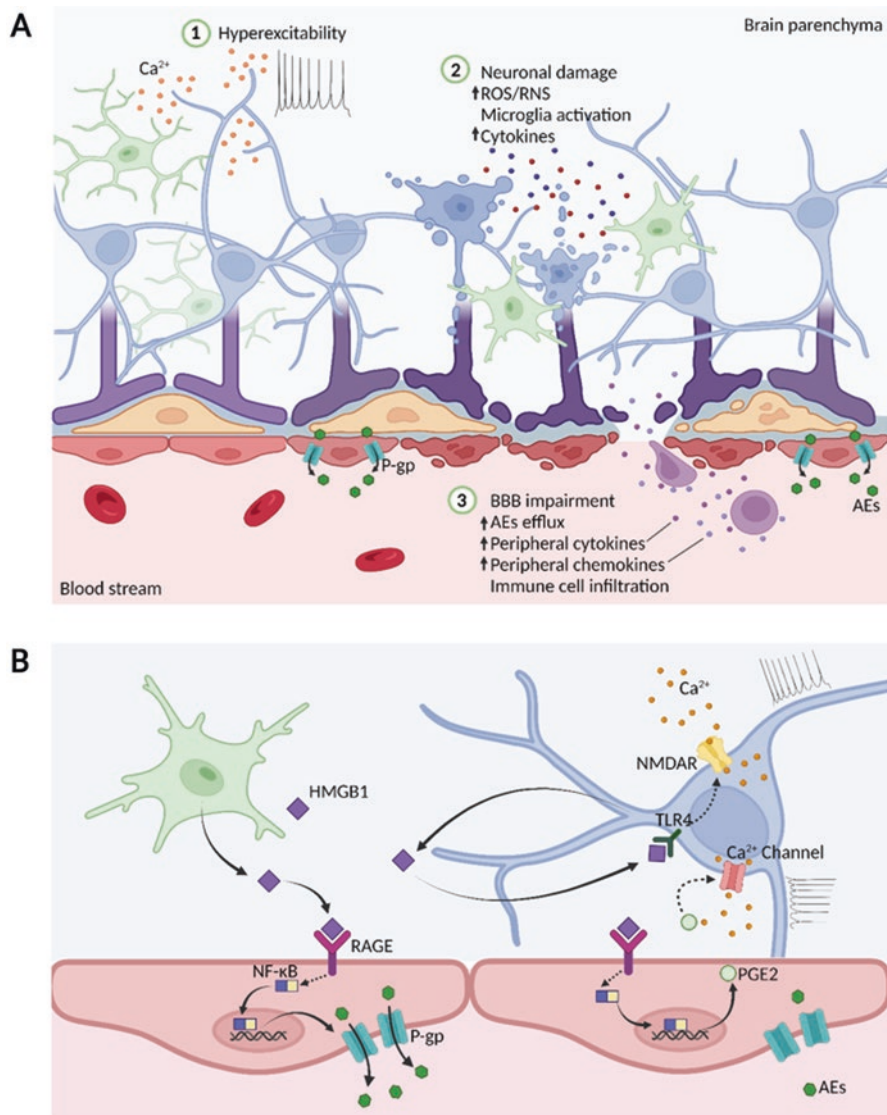


Fig. 8.1 Cellular and molecular inflammatory mechanisms involved in drug-resistant epilepsy. Brain inflammation can originate as a response to peripheral inflammation or a brain injury, both causing neuronal damage. **(a)** The release of DAMPs triggers the innate immune response in the brain through glial cell activation and secretion of proinflammatory cytokines and ROS/RNS that causes neuronal damage and BBB disruption, therefore allowing the access of peripheral immune cells and inflammatory factors. **(b)** Inflammation can promote DRE by mechanisms that include the secretion of HMGB1, which constitutes a natural ligand for RAGE and TLR4. The activation of RAGE by HMGB1 in the endothelial cells of BBB contributes to the nuclear translocation of the transcription factor NF- κ B that induces the expression of P-gp, facilitating the efflux of antiepileptic drugs (AEs) from the brain parenchyma. HMGB1-RAGE signaling in the endothelial cells also induces the production and secretion of prostaglandin E2 (PGE2), which binds to neuronal Ca^{2+} channels increasing intracellular Ca^{2+} and excitability. Furthermore, HMGB1 can activate neuronal TLR4, which promotes the phosphorylation of NMDA receptors (NMDAR), also causing the increase of intracellular Ca^{2+} and excitability. All these mechanisms contribute to the hyperexcitability and hypersynchronization of neuronal circuits that induce seizures

Chen et al. (2015) reported that the treatment with HMGB1 prior to the administration of the excitatory agent kainic acid (KA) in C57BL/6 mice increases the expression of P-gp in brain; conversely, treatment with BoxA (HMGB1 antagonist) decreased P-gp expression. Upregulation of P-gp by HMGB1 in mouse microvascular endothelium was associated with overexpression of TLR4 and RAGE, and the activation of NF- κ B, whose inhibition by LPS-RS, FPS-ZM1 and SN50, respectively, reversed the P-gp increase induced by HMGB1 (Chen et al. 2015). Using luciferase reporter assays, the authors demonstrated that the exogenous expression of NF- κ B p65 increased the promoter activity of the multi-drug resistance 1a gene (ABCB1) encoding P-gp (Chen et al. 2015). These data suggest that HMGB1 contributes to P-gp overexpression in the epileptic brain microvasculature through activation of TLR4/RAGE receptors and NF- κ B, which may directly affect endothelial cells, increasing BBB permeability (Fig. 8.1a and b).

Nass et al. (2020), evaluated the serum levels of HMGB1, metalloproteinase 9 (MMP9), RAGE, and S100 in epileptic patients after 2, 6, and 24 h of bilateral generalized tonic-clonic seizures. They observed that the serum levels of HMGB1 and S100 protein increased immediately after the seizure (Nass et al. 2020). Similarly, Kamasak et al. 2020, compared the levels of HMGB1, TLR4, IL-1 β , IL-1R1, and TNF- α in patients with mild and severe epilepsy; they found higher levels of HMGB1, TLR4, TNF- α , and IL-1 β in the severe epilepsy group than in the control and mild epilepsy groups (Kamasak et al. 2020). These data suggest that the serum levels of these cytokines correlate with the severity of epilepsy and that their increase may be greater in patients with DRE, since TLR4 and HMGB1 are increased in the hippocampal tissue of patients with mTLE-DRE (Maroso et al. 2010). Furthermore, TLR4 activation by HMGB1 in neurons induces seizures through the phosphorylation of the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor and the increase of intracellular Ca^{+2} (Maroso et al. 2010) (Fig. 8.1b).

TLR3 also participates in DRE. This receptor, expressed mainly in astrocytes and oligodendrocytes, is involved in neuronal plasticity (Vontell et al. 2013). It has been observed that TLR3 deficiency reduces the frequency of recurrent seizures due to the decreased activation of microglia and the lack of TNF- α and interferon β (IFN- β) secretion in the pharmacological model of epilepsy (Gross et al. 2017).

Hosseinzadeh et al. 2019 reported that pretreatment with TLR ligands decreases seizure severity in the pilocarpine-induced status epilepticus (SE) in rats. This effect correlated with lower IL-1 β and IL-6 expression and higher IL-10 and TGF- β expression in the hippocampus, which correlated with reduced neuronal hyperexcitability in granule cells of the hippocampal dentate gyrus (DG) (Hosseinzadeh et al. 2019).

In addition, IL-1 β and TLR4 are upregulated in the hippocampal brain tissue of patients with mTLE (Leal et al. 2017). Although the specific pathways triggered by the IL-1 β /TLR4 interaction are unknown, these two molecules have identical intracellular domains activated by similar pathways. Therefore, the IL-1 β /TLR4 interaction is critical for developing neuronal hyperexcitability and increased seizure susceptibility (Maroso et al. 2011; Matin et al. 2015). Furthermore, the

IL-1 β /TLR4 pathway has long-term transcriptional effects on inflammatory genes, including pro-IL-1 and NOD-like receptor protein 3 (NLRP3) through activation of NF- κ B; therefore, it prolongs the neuroinflammatory response and increases seizure susceptibility (Vezzani et al. 2019).

8.2.2 Receptor for Advanced Glycation End Products (RAGE) in Epilepsy

The receptors for advanced glycation end products (RAGEs) are multiligand members of the immunoglobulin superfamily (Grossin et al. 2009). RAGEs are mainly activated by glycation end products, proteins, or lipids that become glycated after exposure to elevated glucose levels, such as those observed in type 2 diabetes (Stern et al. 2002). RAGEs recognize a variety of ligands (Schmidt et al. 2000), including the members of the S100 protein family, HMGB1, amyloid beta, and fibrillar protein aggregates (Leclerc et al. 2010; Taguchi et al. 2000; Yan et al. 2009). RAGE activation prompts multiple signaling pathways such as the Ras-extracellular signal-regulated kinase 1/2 (ERK1/2), a central regulator of cell proliferation, the Rho-Rac-CDC42 pathway, involved in cytoskeletal rearrangements, the stress-activated protein kinase/c-Jun-NH2-terminal kinase (SAPK-JNK) and p38 mitogen-activated protein kinase (MAPK) pathways, involved in growth, differentiation, survival, and apoptosis. These signaling pathways activate transcription factors like NF- κ B, cAMP response element-binding (CREB) protein, or members of the signal transducer and activators of transcription family 3 (STAT3) that induce the activation of inflammatory pathways (Huang et al. 2001; Huttunen et al. 1999, 2002). In addition, this interaction seems to be bidirectional since proinflammatory cues can also increase RAGE expression. This positive feedback generates sustained NF- κ B activation, i.e., a chronic pathophysiological state derived from exacerbated inflammation (Huang et al. 2001).

One of the first works describing the upregulation of RAGE expression in epilepsy was performed in patients with focal malformations of cortical development (Huang et al. 2001). These authors showed an increase in RAGE mRNA and described that the cellular distribution of the receptor was mainly in reactive astrocytes and dysplastic neurons (Zurolo et al. 2011).

Furthermore, Iori and colleagues showed that RAGE activation in the hippocampus contributes to seizures and the pro-ictogenic effects of HMGB1 in a TLE model (Iori et al. 2013). Interestingly, the authors also reported high RAGE expression in hippocampal sclerotic tissue from TLE patients, particularly in pyramidal neurons, reactive astrocytes, and blood vessels (Iori et al. 2013).

In recent years, the link between RAGE and DRE has been the subject of many investigations. Guo and colleagues showed a polymorphism in the RAGE G82S locus in a Chinese population. They observed that the 82S+ genotype and S allele were more commonly expressed in DRE patients compared to nonepileptic subjects (Guo et al. 2016). These results suggest a novel association between genetic variants

in the RAGE and DRE, although further studies are required to confirm this genetic relationship in other populations.

One hypothesis regarding the mechanisms of DRE in epilepsy is the “efflux transporters” hypothesis, which suggests that overexpression of efflux transporters in BBB endothelial cells constantly extrudes antiepileptic drugs, preventing their action in the brain (Loscher and Potschka 2005). This hypothesis was based initially on the observation that several multi-drug transporters are highly expressed in the endothelial cells of DRE patients and the pentylenetetrazol (PTZ) seizure rodent model (Fritz 2011; Potschka 2010).

Interestingly, endogenous ligands for RAGE, such as HMGB1, can regulate P-gp expression throughout the RAGE/NF- κ B signaling pathway (Xie et al. 2017). Blocking the transcription of HMGB1, RAGE, or NF- κ B reduces P-gp expression in the rat hippocampus. These results suggest that the RAGE activation could be involved in DRE by increased ABCB1 gene expression and glycation. As mentioned before, RAGE/NF- κ B signaling can also induce neuroinflammation in epilepsy. Thus, studying the mechanisms involved in modulating the RAGE-induced immune response should provide therapeutic targets to prevent proinflammatory damage. In this regard, the dipeptidyl peptidase IV inhibitor sitagliptin has been shown to have an anticonvulsant effect due to its anti-inflammatory action (Liu et al. 2018). Sitagliptin downregulates the RAGE-JAK2/STAT3 pathway and other chemokines, such as CXCL4 and CXC3R (Liu et al. 2018). Surprisingly, the knockdown of CXC3R diminished the expression of the RAGE-JAK2/STAT3 pathway, suggesting a functional link between CXC3R and this signaling pathway (Liu et al. 2018). Moreover, Badawi and colleagues showed that pentoxifylline, a drug utilized to treat muscle pain in patients with peripheral artery disease, has an anticonvulsant effect by modulating the HMGB1/RAGE/TLR4 signaling pathway. In addition, pentoxifylline treatment reduced the protein levels of HMGB1, TLR4, and RAGE in hippocampal homogenates after PTZ injection (Badawi et al. 2021). Furthermore, blocking RAGEs with monoclonal antibodies (mAb) after PTZ administration has an anticonvulsant and neuroprotective effect (Ping et al. 2021). These data highlight the role of RAGE in inflammation and suggest it could be used as a pharmacological target against DRE.

8.3 Cytokines and Chemokines in the Pathogenesis of Epilepsy

Inflammation is one of the factors that contribute to DRE. Cytokines are the main mediators of the inflammatory and immune response because they regulate cell growth, differentiation, and activity in the brain (Vilcek and Feldmann 2004; Vezzani et al. 2011; Vezzani et al. 2013). Several CNS cell types secrete both pro- and anti-inflammatory cytokines. Cytokines affect neuronal excitability through their ability to alter neurotransmitter functions by modulating receptor assembly,

ion channel insertion, neurotransmitter clearance, and phosphorylation in neuronal membranes (Viviani et al. 2007).

IL-1 β is a proinflammatory cytokine that participates in seizure development by stimulating NMDA currents in neurons and increasing intracellular Ca²⁺. IL-1 β receptor IL-1R1 promotes the phosphorylation of the NR2B subunit of the NMDA-glutamate receptor through the Src tyrosine kinases (Balosso et al. 2008; Viviani et al. 2003, 2006). In addition, IL-1 β has important effects on neuronal excitability mediated by astrocytes. IL-1 β inhibits glutamine synthetase in these cells, reducing astrocyte-mediated glutamate uptake and increasing glutamate release. It also increases astrocytic nitric oxide production, promoting neuronal excitability (Bezzi et al. 2001; Hu et al. 2000; Kigerl et al. 2007; Takeuchi et al. 2006; Vitaliti et al. 2019). Overall, IL-1 β enhances glutamate-induced neuronal excitation and, possibly, excitotoxicity, leading to a lower seizure threshold. Increased extracellular glutamate levels would facilitate the activation of ionotropic and metabotropic glutamate receptors, enabling the generation and propagation of epileptic activity (Fellin et al. 2006; Tian et al. 2005).

IL-1 β is released after TLR4 stimulation by any of its ligands (Kigerl et al. 2007). In turn, TLR4 signaling activates the inflammasome, inducing the release of soluble IL-1 β and HMGB1 through a caspase-1-dependent mechanism (Diamond et al. 2015; Yang et al. 2015). The TLR4-IL-1 β axis is key for inflammation-mediated epileptogenesis since ablating TLR4 signaling decreases the number of seizures in epileptic animals (Iori et al. 2013).

IL-6 is another proinflammatory cytokine associated with the pathogenesis of epilepsy. IL-6 is secreted not only by immune cells but also by microglial cells, astrocytes, and sometimes even neurons, perivascular cells, and brain endothelial cells in response to inflammatory stimuli and neurologic infections (Fabry et al. 1993; Joseph et al. 1993; Munoz-Fernandez and Fresno 1998). Liimatainen and colleagues reported elevated levels of IL-6 in the serum and CSF of epileptic patients after tonic-clonic seizures (Liimatainen et al. 2009), but whether IL-6 is directly responsible for epileptogenesis is still unknown. Furthermore, Toledo and colleagues have described a significant increase in peripheral levels of IL-6 and IL-5 in patients with DRE (Toledo et al. 2021).

The interaction between IL-6 and IL-17 is also important for DRE since IL-6 activates IL-17, a cytokine involved in the positive feedback of activation of T-helper 17 (Th17) cells (Miossec et al. 2009). Th17 cells are highly inflammatory lymphocytes whose activity contributes to the disruption of the BBB in pathologies like multiple sclerosis and autoimmune encephalomyelitis (Balasa et al. 2020). The IL-17/Th17 pathway has also been related to the pathogenesis of epilepsy, since IL-17 is elevated in CSF and serum of patients with epilepsy of unknown origin, suggesting that there may be a correlation between levels of IL-17, seizures, and BBB dysfunction (Kebir et al. 2007; Miossec et al. 2009).

In addition to interleukins, other cytokines can promote seizures through noninflammatory mechanisms. One example is TNF- α , which, instead of triggering a proinflammatory cascade, modulates neuronal excitability through neurotransmitter release and uptake (Pickering et al. 2005; Santello et al. 2011).

Astrocytes secrete TNF- α and have been shown to rapidly induce the expression of AMPA receptors in neurons through the p55-induced activation of PI3K (Beattie et al. 2002). Newly expressed AMPA receptors lack the GluR2 subunit, making them more permeable to Ca²⁺. These changes lead to a significant increase in the frequency of AMPA-induced excitatory postsynaptic currents (Beattie et al. 2002). Furthermore, TNF- α reduces the amount of GABA_A receptors by increasing their endocytosis, lowering the seizure threshold (Stellwagen et al. 2005). This mechanism is similar to that of TGF- β , which influences glutamate release and response (Diniz et al. 2014).

Chemokines are other proinflammatory signals involved in epilepsy. These molecules are small cytokines with chemoattractant properties that guide the directional migration of leukocytes (Hughes and Nibbs 2018). These molecules are classified as CXC, CC, CX3C, or C chemokines based on the position of conserved cysteine residues in their amino acid chain, and they can be homeostatic or inflammatory (Zlotnik and Yoshie 2000).

Inflammatory chemokines are secreted in response to proinflammatory stimuli in pathological conditions to attract immune cells (monocytes, macrophages, T lymphocytes, and mast cells) to the damaged site. Some examples are CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), and CX3CL1 (fractalkine) (Raman et al. 2011).

Chemokines interact with G protein-coupled transmembrane receptors on the surfaces of their target cells. Chemokine receptors are divided into four families based on the type of chemokine they bind to. For example, CCRs bind to CC chemokines, and CX3CR1 binds to CX3CL1. Upon chemokine binding, they can undergo homo-dimerization or hetero-dimerization to activate many signaling cascades, including the Rho-GTPases and MAP pathways (Raman et al. 2011).

Chemokine receptors are not restricted to leukocytes. In the diseased brain, chemokine receptors are also found on microglia, astrocytes, oligodendrocytes, and neurons. Furthermore, chemokine signaling is involved in various CNS pathologies, particularly those with an inflammatory component, such as epilepsy (Raman et al. 2011). These findings suggest that chemokines are key players in the modulation of neuronal excitability, the recruitment of leukocytes, and inflammatory diseases of the CNS. Therefore, it is interesting to evaluate whether these molecules are also involved in the pathogenesis of epilepsy, since chemokines can directly affect neuronal excitability, most likely through their receptors expressed both presynaptically and postsynaptically (Rostene et al. 2011).

Until now, most research on brain chemokines has focused on modulating neuronal activity under physiological conditions. How chemokines regulate neuronal activity in pathologies characterized by altered neuronal activation, such as epilepsy, is a crucial but still an unexplored research topic. In an attempt to identify potential therapeutic targets, several investigations have studied the role of chemokines in neuroinflammation and immunomodulation in different neurological diseases, including ischemic and hemorrhagic strokes, demyelinating disorders, and progressive neurodegenerative pathologies such as Alzheimer's and Parkinson's

diseases (Bagheri et al. 2018; DiSano et al. 2019; Guedes et al. 2018; Y. Lee et al. 2019; Minami et al. 2006; Mracsko and Veltkamp 2014; Zuenä et al. 2019). Studies have shown that, beyond their role in neuroinflammation, chemokines also interact with neuropeptides and neurotransmitters directly, modulating neuronal activity (Cardona et al. 2008; Le Thuc et al. 2015).

Different pharmacological models have revealed the molecular mechanisms whereby chemokines contribute to the onset of seizures. For example, CCL2 expression increases after pilocarpine-induced seizures (Foresti et al. 2009; Manley et al. 2007). In mice with kainate-induced seizures, CCL2 receptor (CCR2) activation initiated the STAT3 signaling pathway, which is involved in neuronal cell death and IL-1 β production (Tian et al. 2017). CCL2 also triggers seizures by altering intracellular calcium signaling and enhancing postsynaptic currents in the hippocampus (Zhou et al. 2011).

In addition, the interaction between CXCL12 and CXCL4 can induce TNF- α secretion from microglial cells, thereby influencing synaptic function and enhancing glutamate release (Lee et al. 2007). High levels of CCR5 (which binds CCL3, CCL4, and CCL5) have been reported in the brains of animals with epilepsy induction, suggesting a possible epileptogenic role (Cerri et al. 2017). Finally, the chemokine CX3CL1/fractalkine can decrease GABAergic inhibitory functions in patients with mTLE through its receptor CX3CR1, expressed by glial cells. This effect was mediated by the reduction of GABA_A receptor currents, suggesting that the GABAergic system is significantly modulated by CX3CL1 released in epileptic foci. These observations open a wide scenario of therapeutic opportunities to control the hyperexcitability of neuronal networks in epileptic patients (Roseti et al. 2013).

Human studies have shown promising results and further emphasize the role of chemokines in epilepsy (Fabene et al. 2010). It is now well known that CCL2, CCL3, and CXCR4 (the CXCL12 receptor) are critical for driving leukocytes to inflamed areas: these receptors are also highly expressed in brain tissue surgically removed from patients with TLE (Arisi et al. 2015; Wu et al. 2008).

8.4 Reactive Oxygen Species and Epilepsy

The brain is highly sensitive to oxidative stress due to its high oxygen consumption, metabolic demand, concentration of polyunsaturated fatty acids, and amount of neuronal mitochondria. The considerable ATP consumption necessary to sustain ionic gradients generates a high metabolic demand in neurons. Most neuronal ATP is generated by mitochondrial oxidative metabolism and strictly depends on oxygen supply (Johnson et al. 2021).

Acquired epilepsies are caused by a precipitating brain injury such as trauma, infections, ischemic stroke, or status epilepticus. These injuries lead to a process known as epileptogenesis, in which biochemical and cellular changes trigger spontaneous recurrent seizures within a latent period of several weeks to years after the first precipitating insult (Devinsky et al. 2018; Eastman et al. 2020). Since

metabolic dysfunction and redox imbalance in neurons may directly impact seizure generation and propagation, it is essential to understand the conditions that exceed the cellular antioxidant system leading to neuronal loss, cognitive impairment, and the spread of epileptic seizures (Geronzi et al. 2018; Johnson et al. 2021).

Oxidative stress is the imbalance between free radicals generation and insufficient antioxidant production (Pizzino et al. 2017). Free radicals are named reactive oxygen species (ROS) when the unpaired atom is oxygen, and reactive nitrogen species (RNS) when the unpaired atom is nitrogen. Oxygen is mainly involved in the generation of energy for cell metabolism and in the oxidation of biological compounds. Regulating the balance of ROS is important for many cellular functions (mainly in microglia and astrocytes), including calcium mobilization, apoptosis, and MAPK cascade activation. Under homeostatic conditions, a certain level of ROS is necessary to respond to the increased mitochondrial energy demand. Nitric oxide (NO) is a biological signaling molecule involved in physiological processes like neurotransmission, defense mechanisms, immune response, and blood pressure. NO reacts easily with superoxide anion (O_2^-) to form the highly reactive peroxynitrite (Geronzi et al. 2018).

Oxidative stress might contribute to neuronal hyperexcitability and neuronal loss through diverse mechanisms, for example:

- (i) Oxidative damage to structural and membrane proteins such as neurotransmitter receptors and ion channels.
- (ii) Structural and functional changes in mitochondria.
- (iii) Cell death (neuronal loss, inflammation, and mitochondrial dysfunction).

In animal models, epilepsy is associated with cognitive impairment and oxidative stress caused by seizure activity. Studies using iron-induced epilepsy in rats and phosphinotricin-treated mice reported the induction of oxidative markers (Collin 2019). Moreover, acute seizures increase ROS, generating an adaptive increase of mtDNA repair; however, chronic ROS production is accompanied by a failure to repair mtDNA (Aguiar et al. 2012; Ethemoglu et al. 2021). Recently, it has been described that several markers of oxidative stress such as malondialdehyde, 4-hydroxy-2-nonenal, and nitrotyrosine were increased in serum samples from DRE patients (Lorigados Pedre et al. 2018). Furthermore, antioxidant systems such as glutathione and SOD are altered in patients with TLE (van Horssen et al. 2019).

The inhibition of ROS generation can ameliorate neuronal damage in seizures. However, antioxidant therapy has been inefficient against seizure development partly because of the short-lived effects of direct antioxidants and their limited passage through the BBB (Yang et al. 2020). A proposed alternative is to increase the endogenous cellular antioxidant defenses by upregulating the transcription factor Nrf2, which provides substrates for mitochondria and ATP production (Shekh-Ahmad et al. 2018). Recently, USP15 inhibition was found to prevent glutamate-induced oxidative damage and neurotoxicity by activating the Nrf2/HO-1 signaling pathway in HT22 cells (Chen et al. 2020); this suggests that USP15 might be a promising therapeutic target to prevent neurodegenerative diseases progression associated with Nrf2 activation (Das et al. 2021; Villeneuve et al. 2013).

8.5 Conclusion

Clinical and experimental studies support the importance of studying the mechanisms of innate and adaptive immunity in the progression of epilepsy and the development of DRE. In particular, the TLRs/IL-1 β , HMGB1, and RAGE signaling pathways suggest participating in DRE, increasing the severity, frequency of seizures, and disease progression. These pathways promote a chronic neuroinflammatory state associated with BBB dysfunction and high oxidative stress, along with the expression of drug-binding proteins such as P-gp, decreased seizure threshold, and neuronal cell death. Although several anti-inflammatory treatment strategies improve the response of anticonvulsant drugs, they do not exert antiepileptic effects. In addition, the mechanisms through which they act have not yet been fully described. Therefore, new translational studies should be aimed at designing pharmacological strategies with immunomodulatory potential that facilitate adequate control of antiepileptic drugs and prevent DRE progression.

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Chapter 9

Contribution of the Antiepileptic Drug Administration Regime to Avoid the Development and/or Establishment of Pharmacoresistant Epilepsy



Pietro Fagiolino and Marta Vázquez

Abstract Although drug-resistant epilepsy has been explained by different mechanisms, the most accepted one involves overexpression of efflux transporters at the blood-brain barrier and at drug-elimination tissues. Tissue expression of efflux transporters can not only be triggered by some antiseizure medications (ASMs) with inducing properties but also by physiological factors such as redistribution of cardiac output. Tissues with high metabolic rates in need of oxygen and glucose will receive a higher fraction of blood and, thus, any other solutes from blood including ASMs. The increased expression of efflux transporters in these tissues due to the increased cardiac output fractions triggered by the energetic tissue requirements will precisely avoid the entrance of any unnecessary substance. Increased expression and function of transporters in the brain are observed in rest periods when there is a significant withdrawal of blood flow from the skeletal muscles and a persistent influx of magnesium. Then, circadian rest-activity rhythms condition not only the expression of transporters but also their functioning.

Even though glucose is the common fuel for the brain, ketone bodies are a much better source of energy. Apart from the ketogenic diets, ketone bodies can be obtained through physical exercise and through the metabolism of long-chain fatty acids via L-carnitine. Besides, physical activity also decreases the cerebral fraction of cardiac output, contributing then to avoiding its high rate of efflux.

Therefore, possible strategies to overcome drug resistance consist of: administering old ASMs under a bitherapy regime, as is the case of valproate and phenytoin (or another drug of choice) but with different dosing intervals; co-administration of L-carnitine, as a supplement to support an adequate metabolism of fatty acids and also to protect the patient from eventual hyperammonemia valproate can cause; and appropriate routine of physical exercise.

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Keywords Efflux transporters overexpression · Circadian rhythms · Tissue blood flow · Tissue metabolic rate · Physical exercise · Therapeutic management

9.1 Refreshing Last Edition of the Chapter and Scope of the Present Update

The main topics covered in the past edition of the chapter (Fagiolino et al. 2013) are summarized here below:

- Overexpression of efflux transporters in the brain, and in tissues that participate in systemic drug elimination, is our main hypothesis to explain pharmacoresistant epilepsy.
- Most of antiseizure medications (ASMs) are substrates of efflux transporters.
- Several ASMs induce the expression of efflux transporters as nonconventional side effects.
- Overexpression of efflux transporters caused by the mere presence of ASMs might enhance (phenytoin) or diminish (carbamazepine) their systemic concentrations, but it always reduces their brain/blood levels ratio.
- Salivary ASM concentrations supported our hypothesis of pharmacoresistance.
- Treatments that sustain ASMs' concentrations, due to frequent drug dosing or the use of sustained-release drug products were the main iatrogenic cause of pharmacoresistance we invoked in the last edition of the book.

In this update, we will focus on the physiological factors that trigger the tissue expression of efflux transporters and what measures should be taken in order to modulate their incidence in conjunction with modifying the drug dosage regime.

Some data allow us to relate tissue expression of efflux transporters to the fraction of cardiac output received by each tissue, providing a strong rationale for developing drug-resistant epilepsy once seizures become persistently uncontrolled. The following statements summarize the concept:

- ATP-binding cassette (ABC) transporters are expressed in healthy tissues which receive the highest fraction of blood flow with respect to their sizes: lungs, liver (and intestine), kidney, heart, and brain, among others.
- Systemic organs having high metabolic rates receive a larger fraction of the cardiac output in order to increase the uptake of oxygen.
- The higher the fraction of cardiac output a tissue receives, the higher the oxygen uptake is and, consequently the oxidative stress suffered by its cells.
- Repeated seizures lead to higher oxygen consumption with the subsequent oxidative stress of the brain and thereafter, the establishment of chronic inflammation.
- The increase in the brain fraction of cardiac output not only increases the uptake of oxygen and glucose (preferred fuel of brain) but also the uptake of any other solute from blood, like ASMs.

- Efflux transporters have precisely the function of detoxifying the cells of toxic metabolites derived from oxidative stress and of unnecessary xenobiotics (drugs) for their vital machinery.

Ultimately, these physiological conditions would explain pharmacoresistant epilepsy, which subsides momentarily when effective ASMs are administered. However, drug resistance is restored over time if these ASMs are inducers of efflux transporters or if the causes of brain excitotoxicity are not abolished (i.e., hypoxia, excitatory agents, ammonia, etc.).

Some time ago, Arida et al. (2010) showed how physical activity improves anti-seizure treatment. In addition to the reasons the researchers invoked, we believe that the reduction in the cerebral fraction of cardiac output caused by physical activities contributes significantly to reducing cerebral oxidative stress and, consequently, to lowering the rate of efflux transporters expression. Besides, the formation of ketone bodies during exercise provides an alternative fuel to glucose that generates greater production of ATP in relation to the volume of oxygen consumed and contributes to reducing oxidative stress.

How to combine physical activity with the administration of drugs that could even induce the expression of transporters (phenytoin, carbamazepine), or with drugs whose side effects include the formation of proconvulsant substances (hyperammonemia provoked by valproate) is what this new version of the chapter proposes. In addition, it will go deeper into physiologically based pharmacokinetic principles linked to the blood flow that a tissue receives, to explain the transfer of molecules from the bloodstream to the extravascular space of tissues.

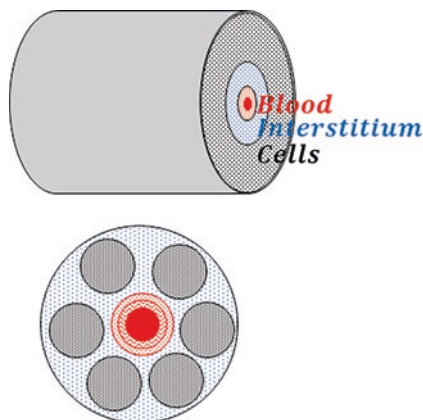
9.2 The Effect of Cardiac Output Distribution on Tissue Drug Concentration

9.2.1 Body Water Distribution

Male subjects of 70 kg weight have 5 L of blood (B), which means around 4.5 L of water in the intravascular (IV) space. Within the extravascular (EV) space, 9 L of water are located in the interstitium, whereas the tissue (T) cells contribute 27 L of water. Mixed blood (pulmonary circulation and systemic arteries) comprises 31% of the total blood, whereas nonentirely mixed blood (systemic veins) represents 64%. Capillaries in the systemic circulation contain the rest 5% of the total blood.

Stroke volume (~80 mL) represents one-third of the blood placed in the capillaries (~250 mL) of the systemic circulation. Then, after each systole, one-third of the capillary blood pushes ahead of the vessels (red circle shown in Fig. 9.1), the volume that is drawn from the capillary bed during diastole, another one-third of blood pushes against the endothelial walls of capillaries and the last one-third remains practically without moving along the endothelial membrane in the so-called boundary layer (shown in Fig. 9.1 as rings embedded with red waves or red dots,

Fig. 9.1 Capillary and surrounding space. Water distribution between the different intra (blood) and extravascular (interstitium and intracellular) compartments. The intracellular water actually disperses into the interstitial water, as shown at the *bottom*



respectively). From this layer, solutes within the blood migrate toward the EV space by diffusion. Solute in the other two-thirds of the capillary blood move by convection (solute plus solvent). In fact, solute transportation throughout the vessels of the circulatory system and the interstitial space of tissues is mainly by convective movement while diffusion is the main mechanism by which solutes cross the capillary endothelium and the rest of the tissue cell membranes (Hladky and Barrand 2014).

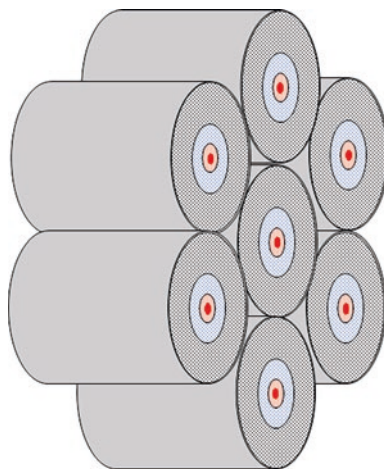
Figure 9.1 outlines the distribution of water in the different tissue spaces: intravascular, interstitial, and intracellular. A cross-section of the capillary and its adjacencies shows how the tissue cells increase the intracellular space's surface area in contact with the interstitial space. In this way, the interstitium-cell exchange of solutes becomes faster. Figure 9.2 shows each capillary bed as a group of capillaries with their respective surrounding extravascular spaces, while several capillary beds would make up each tissue. This in turn causes solutes to be exchanged very rapidly between blood water and tissue cell water.

This ingenious way of compartmentalizing body water allows the oxygen and nutrients, that each cell needs for its vital processes, arrive as quickly as possible, in the same way that the waste from such processes is cleared out of the body. As it will be analyzed in the following sections, the kinetics of exchange between the different aqueous spaces of the body occurs so quickly that the mere adjustment of the working conditions of the cardiovascular system will efficiently control the distribution of solutes between the tissues.

9.2.2 Tissue Metabolic Rate and Tissue Blood Flow

Energy tissues demand to support their metabolic rate is linked with their oxygen and fuels consumption (Wang et al. 2010; Jacob et al. 2016). Considering that solutes in the arterial blood are delivered to the tissues at the same concentration,

Fig. 9.2 Each capillary bed is made up of several capillaries and their surrounding extravascular spaces. In turn, tissues are made up of several capillary beds



independently of their metabolic rate, tissue blood flow, as a fraction of the cardiac output, plays a fundamental role in their respective solute uptakes. The higher the fraction of cardiac output a tissue receives, the higher the oxygen and solute uptake (Fagiolino 2002; Fagiolino et al. 2003; Fagiolino and Vázquez 2022).

Tissues differ from each other by the morphology and function of their cells, besides their nonaqueous structure content. However, considering that no cell in the body is more than two cells away from a capillary, the water content in the surrounding EV spaces of tissue capillaries could be assumed to be similar. The single difference between tissues is the cardiac output fraction that each of their capillaries receives. In other words, tissues with higher metabolic rate, and hence, irrigated with a higher blood flow in relation with their EV water content, will have higher volumes of blood with convective motion, and then, lower nonconvective volumes (boundary layer: outer ring of capillary blood volume shown in Fig. 9.1). A reduction in the thickness of the boundary layer determines an increase in the transfer rate toward the extravascular space of the tissue.

Oxygen delivery is closely regulated by the diameter of the arterial blood vessel. If a tissue requires more oxygen due to intense metabolism, local vasodilation proceeds at expense of vasoconstriction in another tissue with a lower metabolic rate. However, there is a limit to reducing the relative blood volume received by a tissue (survival threshold for the cardiac output fraction). In case vasoconstriction compromises the survival of a given zone of tissue, other zones from the same or from different tissues must contribute to supplying the oxygen requirement of a highly demanding area of the body. Fortunately, not all body cells simultaneously demand a high proportion of the oxygen the arteries carry.

Human body knows this basic rule very well. At rest, two-thirds of body tissues, having lower metabolic rate, supply the cardiac output fraction required by the other one-third having higher metabolic rate. As it will be discussed later, physical exercise drastically changes this distribution pattern of cardiac output, leading to a more equal distribution, although it is not sustainable for the long term.

9.2.3 Kinetics of Solute Exchange Between Blood and Tissues

If the volume of blood ejected by the heart during each systole were distributed equally to all the capillaries of the body, the boundary layer of blood at each capillary would have the same thickness, and therefore the transfer of solutes to the EV space of tissues would proceed with the same rate. Kinetically speaking, blood-to-tissue solute clearance has the same value regardless of the capillary endothelium considered. This means that each tissue receives a cardiac output fraction (σ_i), which is proportional to the surface area of its whole capillary endothelium. Equation 9.1 relates the individual blood-to-tissue clearance with the total blood-to-tissue clearance for a given unbound and nonionized solute

$$CL_{\text{blood-tissue}i} = \sigma_i \cdot CL_{\text{blood-}\Sigma\text{tissues}} \quad (9.1)$$

Since the EV/IV water/water partition coefficient of any unbound and nonionized solute is 1, the total clearance back to blood equals the total blood-to-tissue clearance (Eq. 9.2)

$$CL_{\Sigma\text{tissues-blood}} = CL_{\text{blood-}\Sigma\text{tissues}} \quad (9.2)$$

As clearance is proportional to the endothelium surface area and this is also proportional to the EV water a tissue has, each individual tissue-to-blood clearance coincides with the total tissue-to-blood clearance multiplied by the fraction (ω_i) of the EV body water it contains (Eq. 9.3).

$$CL_{\text{tissue}i\text{-blood}} = \omega_i \cdot CL_{\Sigma\text{tissues-blood}} \quad (9.3)$$

At the equilibrium, that is, when the input of solute equals its output from the body, the tissue/blood concentration ratio is given by Eq. 9.4. This equation means that the tissue/blood ratio (γ_i) equals 1 only if the fraction of the cardiac output served to the tissue equals its fraction of EV body water, representing that the stroke volume is distributed equally to all the capillaries of the body

$$\frac{C_{\text{tissue}i}}{C_{\text{blood}}} = \frac{\sigma_i \cdot CL_{\text{blood-}\Sigma\text{tissues}}}{\omega_i \cdot CL_{\Sigma\text{tissues-blood}}} = \frac{\sigma_i}{\omega_i} = \gamma_i \quad (9.4)$$

However, as explained in the previous section, tissues have different metabolic rates and therefore receive different fractions of the cardiac output. This implies that the boundary layers of the capillary blood do not have the same thickness, and consequently each blood-to-tissue clearance will be different, although its respective tissue-to-blood clearance remains unchanged.

This means that the tissue/blood solute concentration ratio will no longer equal 1. This is not because of a change in the EV/IV water/water partition coefficient at the blood-tissue interface (capillary endothelium), but due to the mix of blood coming from all body capillaries. In other words, due to the mix of all the blood

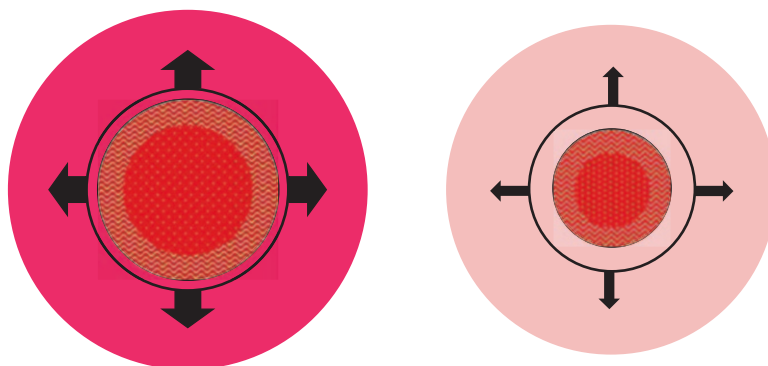


Fig. 9.3 Solute transfer from blood water to tissues' extravascular water (represented by *black arrows*) actually takes place from the blood boundary layer (*outlined by curved black lines*). In fact, the water/water partition coefficient equal to 1 is just verified at the extravascular space/boundary layer interface. Note that the tissue concentration of solute is greater (more intense color in the *left panel*) when the thickness of the boundary layer is reduced as a consequence of the higher fraction of cardiac output received by the capillary

volume with convective movement. Only from this portion of the blood, the unbound nonionized solute concentration is able to be measured. As for oxygen and cell fuels or nutrients, this also applies to ASMs once they reach the steady state concentration after following their respective dosage schedules. Figure 9.3 illustrates the role of the boundary layer thickness (framed by black curved lines) at the capillary endothelium in the blood-to-tissue clearance (black arrow size). When it is reduced or augmented, tissue solute concentration increases or decreases (left or right panel, respectively), even though its partition coefficient between the extravascular water and the boundary layer of blood water remains constant (Fagiolino and Vázquez 2022).

Boundary layer concept comes from the mass and heat transfer theory for fluid flowing in contact with a solid surface (Welty et al. 1996; Rocha-Uribe et al. 2021). Under the laminar flow regime, fluids flowing through pipelines develop at the adjacencies of the cylindrical wall, a narrow zone where the axial motion is less than that in the center of the cylinder. Very narrow at the beginning, and then it widens as it progresses throughout the pipeline (Fig. 9.4). The increased pressure against the endothelial wall at the beginning of the capillary generates the driving force for solute transfer toward the extravascular space. As the boundary layer widens, the transfer wanes. Some researchers suggest that this high pressure on the capillary endothelium would cause the permeation of water even in barriers as restrictive as the blood-brain barrier (Hladky and Barrand 2014), thus leading to an increase in solute concentration at the boundary layer and, therefore, a greater diffusion toward the tissue. Aquaporins 1 and 4 (AQP1 and AQP4), members of a family of membrane water channels, are known to be expressed in the central nervous system, and it is possible that these proteins contribute to water transport across the blood-brain barrier (Bonomini and Rezzani 2010).

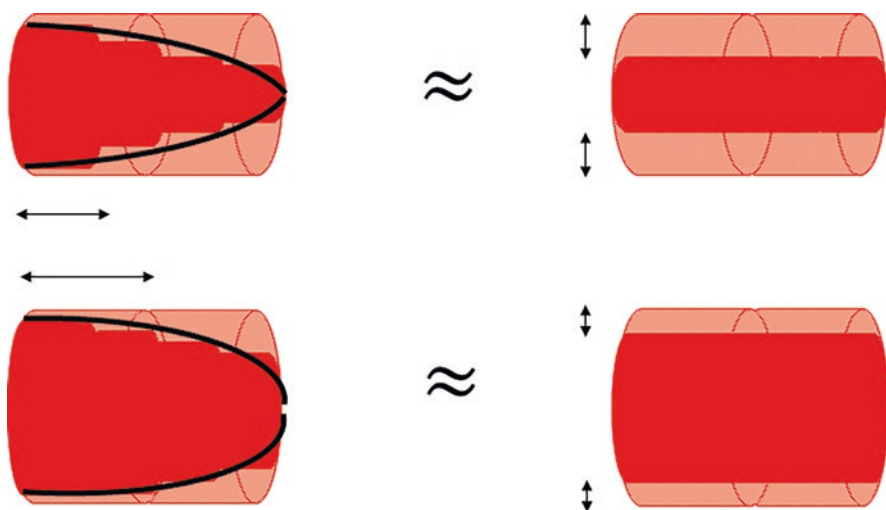


Fig. 9.4 Blood flowing through the capillary according to the laminar flow model. The higher the cardiac output fraction, the longer the boundary layer developed at the capillary wall (*left panel*). In the *right panel*, this is represented as a narrower boundary layer along the entire capillary wall, as it was referred to in Fig. 9.3 under a cross-section view

A higher fraction of cardiac output delivered to the tissue causes a lengthening of the narrow boundary layer along the capillary (longitudinal recruitment), thus causing a greater uptake of solutes by the tissue. The boundary layer of constant thickness shown in Fig. 9.3 just represents a simplification in order to manage kinetically such a complex biological phenomenon. Since the solutes contained in the compartment shorten their distance to the exit interface, the rate of transfer increases (Fagiolino 2004). The boundary layer concept is now becoming very useful in explaining the uptake of solutes by different kind of tissues (Nishihara and Ackerman 2007; Lichtenberg et al. 2017). Besides, some investigations show the importance that a pulsatile flow could have in the uptake of oxygen (Moschandreou et al. 2010).

Certain tissues (liver, intestine, and kidneys) concentrate within their cells a significant number of enzymes that make them specialized in eliminating xenobiotics (like ASMs). However, even so, these enzymes make up only one in a thousand of the hepatocyte mass. Therefore, the elimination clearance of xenobiotics represents a very low proportion in relation to their return clearance to the blood, since the surface area exposed by enzymes is much smaller than that of cell membranes. Then, even at the elimination tissues, the tissue/blood free drug concentrations ratio is ruled by Eq. 9.4; therefore, overexpression of metabolizing enzymes will just reduce drug concentration in both the tissue and blood without any change in such ratio. In order to maintain a high efficiency in the elimination of xenobiotics, their intracellular levels should not be reduced, since their elimination rate is proportional

to such concentrations. This is the reason why our body avoids reducing the intestine or liver or kidney/blood concentration ratios of xenobiotics.

9.2.4 Blood Flow Fraction and Efflux Transporter Expression

As mentioned in Sect. 9.1, efflux transporters are much more expressed in tissues that receive the highest fraction of cardiac output due to their higher metabolic rates, although they do not have the highest fraction of body water. According to what was previously discussed, their tissue/blood solute concentration ratio should be high. However, due to the action of efflux transporters, which are placed in both the tissue cell membranes and the luminal membranes of the capillary endothelium, tissue-to-blood clearance increases reverting; in this way, the effect a high fraction of blood flow causes. In fact, efflux transporters have a protective role against xenobiotics (Epel et al. 2008), which could be unnecessarily introduced into tissues due to the increased cardiac output fractions triggered by their energetic requirements. This protective role becomes evident during brain maturation (Strazielle and Ghersi-Egea 2015) already from the earliest embryonic stages (Kohen et al. 2019).

In view of this, Eq. 9.4 must be adjusted (Eq. 9.5) by means of another factor (μ_i) that refers to the membrane capacity for decreasing the entry of solutes (efflux transporters, $\mu_i < 1$) or even for increasing it (influx transporters, $\mu_i > 1$)

$$\frac{C_{\text{tissue}i}}{C_{\text{blood}}} = \gamma_i \cdot \mu_i \quad (9.5)$$

Mostly, influx transporters allow the passage of molecules across the membrane in both directions. However, efflux transporters have unidirectional effect, mainly from the inner of cells toward the interstitium (membrane of tissue cell), toward the blood (capillary endothelium), and even toward the exterior of the body (apical membranes of intestinal, hepatic, and renal cells).

Seizures provoke a high energy expenditure of neurons, a loss that must be recovered by increasing the oxygen and glucose uptake by increasing blood flow fraction (Duncan 1992; Theodore et al. 1996). Based on this, it is easy to predict the overexpression of efflux transporters in brain areas with high neuronal excitation. An inflammatory mechanism seems to be the activator of such overexpression (Grewal et al. 2017) in response to oxidative stress suffered by tissue cells due to the intense influx of oxygen and subsequent formation of reactive oxygen species. If seizures become repetitive and uncontrolled for a long time, refractoriness to ASMs is installed. It has been postulated that efflux transporter overexpression extends as far away from the brain as xenobiotic-scavenging tissues, such as liver and intestine (Lazarowski et al. 2007; Czornyj et al. 2018). All this together leads to a decrease in the systemic concentration of ASMs, but above all, to a reduction in their brain/blood concentration ratio (Vázquez and Fagiolino 2022).

9.2.5 Circadian Rhythms of Blood Flow Fraction and Efflux Transporter Activity

The circadian rhythm can be divided into two parts: the central clock, residing in the suprachiasmatic nucleus of the hypothalamus, and the peripheral clocks residing in various tissues throughout the body. The main stimulus for the central clock is light, however a varied series of cues could be acting as stimuli. Simplifying, we could say that living beings have a time to eat and a time to sleep. Throughout the day, some signals initiate a particular cycle at each tissue: providing food, either by getting it from nature or by working in order to raise money to buy it (skeletal muscles); eating and digesting it (digestive tract and liver); processing and storing the nutrients (liver, adipose tissue, etc.); eliminating the waste product of cellular metabolism (liver, kidneys, etc.). Appropriate breaks during the day, and fundamentally sleeping at night, allow the brain, the main coordinator of daily activities, to recover its energy level and purge toxins. All these activities determine different patterns of cardiac output distribution in order to assist the correct functioning of the involved tissues.

Cardiovascular activity at each of these times plays then a very important role. It is essential, however, to distinguish between cerebral blood flows (CBF) and cerebral fraction of cardiac output (CFCO) to understand the impact of cardiovascular activity on brain solute uptake. Practically all scientific articles (either in humans or animals) refer only to blood flow as a determining parameter for glucose and oxygen uptake (Vaishnavi et al. 2010; Hodkinson et al. 2014), but as it was mentioned in the previous section, CFCO is what really determines brain uptake.

During sleep, both heart rate (cardiac output) and CBF decrease (Wauschkuhn et al. 2005); however, the latter decreases to a lesser extent. That is, CFCO increases during the rest period. During REM (rapid eye movement) sleep, both CBF and CFCO significantly increase (Tsai et al. 2021). Due to this increase in CFCO, it is easy to envisage a greater expression of efflux transporters in order to protect the brain from the increased uptake of xenobiotics during rest periods (Pulido et al. 2020). The reason for such an increase during the rest period is the significant withdrawal of blood flow from the skeletal muscles.

The expression of efflux transporters is downregulated by melatonin (Liu et al. 2021), explaining thus its synergism with ASMs (Gupta et al. 2004). The hormone secretion increases soon after the onset of darkness (Grivas and Savvidou 2007), peaks in the middle of the night, between 2 and 4 a.m., and gradually falls during the second half of the night, where REM sleep prevails.

The mechanism that causes an increased efflux activity at the blood-brain barrier does not seem to be an exclusive consequence of the increased expression of the transporter (Pulido et al. 2020), but also an increased influx of magnesium due to the increased CFCO during rest (Zhang et al. 2021). The functional unit of ABC transporters consists of two nucleotide-binding domains that hydrolyze ATP in a magnesium-dependent manner (Seelig 2020). This increased influx of magnesium could be attributed to the expression of the transient receptor potential melastatin

channel 7 (TRPM7) in the blood-brain membrane, which increases from the beginning of the rest period, reaching its maximum point at the beginning of the activity period (Zhang et al. 2021). This pattern of channel expression coincides with the gradual increase in magnesium concentration at the blood boundary layer of the capillary endothelium due to the increase in CFCO mentioned above. It seems that both channels that facilitate the passage of ions and transporters that facilitate the passage of nonlipophilic fuels (glucose) match their massive arrivals at the boundary layer of the capillary endothelium.

In short, the cellular biochemistry of tissues would work rhythmically following the orders of their respective peripheral clocks, which prepare the machinery that will be activated by certain actors, from among which, magnesium stands out (van Ooijen and O'Neill 2016; Feeney et al. 2016), being the distribution of cardiac output the driving force.

9.3 Epilepsy and Its Refractoriness

9.3.1 *Seizure Cause*

Stress suffered by a cell can be defined as a disturbance in its homeostatic balance, with which the cell attempts to cope (stress response). Stress can be acute, i.e., immediate response to the stressor, or chronic, i.e., a state caused by a constant stress stimulus. Adequate oxygen supply is needed by the brain to metabolize glucose as its major energy source. Brain function depends critically on an adequate energy supply, and it is highly susceptible to hypoxic conditions (Auzmendi and Lazarowski 2020). A decrease in the concentration of oxygen in the interstitial space of the brain has been observed prior to the onset of seizures (Ingram et al. 2014; Wei et al. 2014) as a consequence of intense interneuron activity, which leads to extraordinary energy expenditure in recovering membrane potentials that cannot be sustained due to an inadequate supply of oxygen. Therefore, endogenously generated hypoxia would be not only the consequence of increased cellular excitability but also a critical factor for orchestrating the epileptiform activity.

Ictal events are preceded by preictal vasoconstriction of blood vessels in the surround, occurring few seconds before seizure onset, which may serve to actively shunt oxygenated blood to the imminently hypermetabolic focus or may be due to small local decreases in metabolism in the surround (Zhao et al. 2011; Volnova et al. 2020). This pathophysiological behavior seems to be related to an altered vascular anatomy that disturbs the cerebral microvasculature (van Lanen et al. 2021).

Debates about the precise cause of triggering seizures still persist: whether tissue hypoxia is because of increased cellular excitability or because of vasoconstriction. However, no one doubts that there will be intense postictal vasodilation in the affected area in order to supply oxygen and fuel to replenish the significant energy expenditure caused (Duncan 2004; Volnova et al. 2020), which in turn also leads to

hypoxia due to the increased uptake of oxygen from blood and its fast consume by neurons.

9.3.2 *Seizure Consequence*

As explained above, increasing the fraction of blood flow delivered to the ictal sites triggers the expression of efflux transporters, which is maintained over time if seizures recur. Not only do efflux transporters increase but also their function is due to an increased and persistent influx of magnesium.

High oxygen and glucose uptake in tissues due to increased blood flow fraction contributes to the development of oxidative stress (Cobley et al. 2018), leading to inflammation (Soomro 2019) and increased expression of efflux transporters (Löscher et al. 2020). Oxidative stress, inflammation, and efflux transport are then closely linked (Cárdenas-Rodríguez et al. 2013). Even worse, chronic neuroinflammation is associated with blood-brain barrier dysfunction due to the induction of the vascular endothelial growth factor (VEGF) (Löscher et al. 2020), one of the main intermediate identified in the genesis of seizures (van Lanen et al. 2021), thus closing a pernicious circle that could only be interrupted with the help of ASMs.

9.3.3 *Refractoriness*

The correct administration of ASMs requires a fine adjustment of their concentrations, not in the blood but in the brain. Due to the overexpression of transporters caused by such frequent seizures, this type of epileptic symptom becomes difficult to control since the usual monitoring of blood drug concentration does not adequately reflect its brain levels. Desperate attempts to increase drug doses fail because many of the ASMs themselves are inducers of efflux transporters. Then, their brain/blood concentration ratios decrease even more (Vázquez and Fagiolino 2022). It can be concluded that epilepsy becomes pharmacoresistant, not because drugs lose their efficacy but their effectiveness. Therefore, given that the efficacy of such drugs would remain intact, the suggested strategy to overcome such alleged refractoriness is to space out higher doses so as not to increase the dosage rate, thus avoiding seizure recurrence and increasing brain drug uptake as well (Fagiolino et al. 2013). Conversely, low oscillation of phenytoin blood concentrations, as a result of frequent dosing, enhances its inducing effect on the expression of efflux transporters (Fagiolino et al. 2017), thus restricting its entry to the brain.

A single high daily dose of phenytoin before going to bed allows not only its good entry into the brain during the first half of the night (higher cardiac output fraction without the overexpression of efflux transporters that will occur late in the night) but also provides adequate concentrations to attenuate the onset of seizures

along the following morning, and hence, reducing the brain's demand for a high fraction of blood flow during daytime. In a certain way, this therapeutic measure resynchronizes the rhythm of the cardiac output distribution, thus fulfilling the paradigm: a time for physical activity and food intake and a time for rest.

Other actions can contribute not only to recovering the patient's normal rhythm but also to reducing oxidative stress in the brain. Some alternative-to-glucose fuels are more efficient producers of ATP (Jensen et al. 2020). In fact, it turned out that ketone bodies (and medium- or short-chain fatty acids) are a much better energy source for the brain than glucose because they produce more ATP per molecule. Besides, ketone bodies regulate reactive oxygen species balance. Apart from providing adequate diets with such fuels (Romano et al. 2017; Fei et al. 2020; Poff et al. 2021), there is another way to generate them and that is through physical exercise.

9.4 Role of Physical Activity in Attenuating Seizure Occurrence and Its Refractoriness

Physical exercise reinforces the normal circadian rhythm of cardiovascular function, since blood is distributed more intensively to skeletal muscles during daytime, reducing significantly the fraction of cardiac output in other body tissues. This also occurs in the brain, although the average cerebral blood flow may keep constant (Joyner and Casey 2015). Nevertheless, not all brain regions share the same fraction of blood flow (Delp et al. 2001; Hayashi et al. 2012). An internal redistribution of blood flow is explained by vasoconstriction in some areas and vasodilation in some other specific areas of the brain due to their enhanced neuronal activity (Delp et al. 2001). Perhaps this process of intracerebral blood redistribution carried out routinely develops neuronal plasticity and brain resilience to deal successfully with seizure-triggering events (Arida and Teixeira-Machado 2021).

Among different mechanisms proposed to rationally explain the benefit of physical exercise (Arida 2021; Arida et al. 2021), the attenuation of brain oxidative stress that is achieved by lowering the cerebral fraction of cardiac output becomes as a key element of our approach. Not only because the normal circadian rhythm is resumed, but also because physical exercise provides those efficient fuels supplied by ketogenic diets without the need to implement them (Koeslag 1980; Schraner et al. 2020; Takahashi 2021; Wu 2021).

In order to facilitate the metabolism of long-chain fatty acids to yield ketone bodies and medium- and short-chain fatty acids, L-carnitine supplement becomes a good therapeutic support (Maldonado et al. 2020). Its deficiency has been associated with severe seizure disorders (Kumar et al. 2015), which were reversed or prevented after its administration (Maldonado et al. 2017; Hussein et al. 2018).

9.5 Combined Strategy with ASMs, Dietary Supplement, and Physical Exercise

As a general rule, treatment of epilepsy should be started with a single, appropriately chosen ASM, and combination therapy should be reserved for patients refractory to two or more sequential (or alternative) monotherapies (Löscher and Klein 2021). Despite the availability of over 20 ASMs for symptomatic treatment of epileptic seizures, about one-third of patients with epilepsy have seizures refractory to pharmacotherapy, even under polytherapy (Löscher et al. 2020).

At this point, and following the line of reasoning developed here, the proposed strategy is to carry out a pharmacotherapeutic plan with recognized effective drugs, accompanied by dietary supplements and a routine of physical exercises adapted to each patient. The main objective is to reduce the incidence and frequency of seizures in a sustainable way, with more physiological than pharmacological measures.

As few drugs as possible are recommended in order to treat epileptic patients, preferably those that do not modify the efflux transport. Knowing the innate capacity of our tissues to remove xenobiotics from their cells, even though they might be beneficial, i.e., ASMs, there are practically no synthetic drugs that are not substrates of efflux transporters. If they are inhibitors or inducers of efflux transporters, they should be given under dose regimes that modulate their actions. Once-daily regimes of immediate-release formulations seemed to reduce their effect on efflux transport with respect to its frequent dosing or the use of sustained-release forms.

One of the most commonly used ASMs in combination with other drugs is valproic acid (Fattorusso et al. 2021), which has not been reported to induce or inhibit efflux transporters. However, a high concentration of valproate in the body has been shown to deplete L-carnitine, causing hyperammonemia followed by seizures. Apart from its benefit on long-chain fatty acid metabolism, this side effect reinforces the need to supply the patient with L-carnitine (Maldonado et al. 2017).

Therefore, valproic acid, whether under immediate or sustained-release formulations, plus L-carnitine, becomes the first choice as one of the ingredients in a combined antiseizure bitherapy. Its administration should be done in the morning, 12 hours later than the other ASM given at night (i.e., phenytoin). Hence, a twice-daily administration regime using just two ASMs, each given every 24 hours, summarizes our proposal. Appropriate doses of both ASMs should be adjusted individually based on the patient clinical response and their blood concentrations.

9.6 Conclusions

Overexpression of membrane transporters is one of the mechanisms that promotes drug resistance in the treatment of epilepsy. This process of overexpression occurs spontaneously in response to seizure activity when seizures are poorly controlled. The administration of ASMs, which are substrates of efflux transporters, is prone to

eventual therapeutic failure if seizures are not controlled. The usage of such agents, which also possess the ability to induce the expression of these transporters, leads to the consolidation of refractory epilepsy, as the increase in transporter expression is such that it turns the antiseizure agent into a real shield against the resolution of the seizures.

In this update, we showed how blood flow distribution among body tissues is involved in the activity of efflux transporters. Circadian rhythm of cardiac output distribution is a determinant for activating the already expressed efflux transporters due to tissue magnesium influx and efflux. Membrane transporters are physiologically more active late in the night due to the increased influx of magnesium. The diurnal physical activity reinforces this day-night rhythm by driving blood to skeletal muscles and thus decreasing the cerebral fraction of cardiac output. Besides, physical activity promotes the use of ketone bodies by the brain, hence, correcting some deficiencies in the glucose uptake and/or hypoxia events that normally causes seizures in epileptic patients. L-Carnitine is a good supplement to reinforce the metabolism of fatty acid and the formation of ketone bodies in the liver.

Transporter hypothesis of drug resistance has triggered efforts to develop approaches to overcome enhanced efflux transport at the blood-brain barrier (BBB). These approaches include modulation of transport function, blocking the signaling pathway that up-regulated the transporters in response to seizure activity, bypassing BBB transporters by encapsulation of antiseizure drugs in nano-sized carrier systems, or intracerebral administration. But none of these novel approaches has focused on avoiding or modulating the inducing capacity of the ASMs, modulating blood flow distribution along the day, and promoting the natural production of ketone bodies as a more efficient source of energy for the brain. Some approaches that attempt to circumvent circadian variations in the cardiac output distribution by administering ASMs by nasal route (Musumeci et al. 2019; Goncalves et al. 2021) or their co-administration with melatonin (Fic et al. 2017) are some interesting exceptions.

On the assumption that a drug is especially effective for a certain type of epilepsy, its continuous administration so as to maintain constant levels of the active ingredient, as a therapeutic agent and as an efflux transporter inducer, is a therapeutic problem. Consequently, a less frequent dosing regimen in time is proposed in order to obtain; therefore, lower drug concentrations by the second half of the dosing interval that would allow a downregulation of the over-expression of efflux transporters and; in this way, the following dose of the antiseizure drug is once again effective. Dosing of effective ASMs, even inducers of efflux transporters, should be done before bedtime when brain blood flow fraction is higher, and efflux transporters are not fully expressed yet because of the action of melatonin during the first half of the night. Noninducer ASMs, like valproic acid, should be given in the morning under immediate-release or even better under sustained-release formulations to avoid high peak concentration and thereafter, hyperammonemia development (seizure trigger). In any of the cases, the co-administration of L-carnitine, as a supplement to support an adequate metabolism of fatty acids, would also protect the patient from such an eventuality.

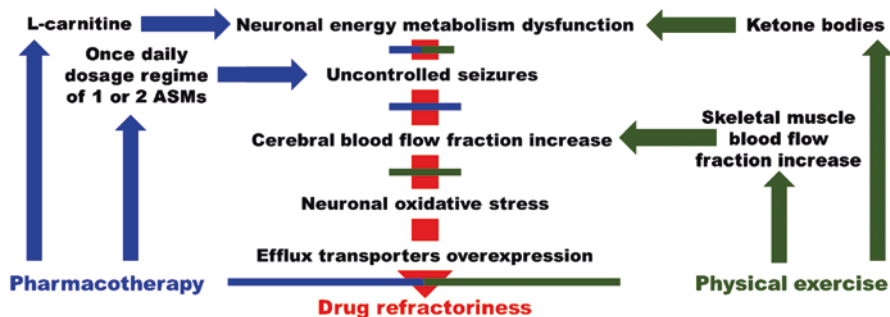


Fig. 9.5 Main attack points of combining physical exercise with the administration of drugs in the fight against refractory epilepsy

In summary, correct ASMs and dietary supplements in appropriate dosage regimens, plus physical exercise plans adapted to each patient, will lead not only to a significantly reduced occurrence of seizures but also to revert the refractoriness to the epilepsy treatment with reasonable success. Figure 9.5 summarizes the main attack points of this combined therapeutic proposal.

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Chapter 10

Pharmacogenetics in Epilepsy and Refractory Epilepsy



Liliana Czornyj, Jerónimo Auzmendi, and Alberto Lazarowski

Abstract Pharmacogenomic studies in epilepsy are necessary due to the high prevalence of this disease and the high percentage of drug resistance phenotype. Several altered genes encode proteins involved in drug metabolism and drug transport (pharmacokinetics), function and expression of receptors, ion channels, metabolic regulatory proteins, or secondary messengers of signaling pathways (pharmacodynamics).

Despite the vast spectrum of antiseizure medications (ASMs) developed to date, refractory epilepsy remains consistently prevalent (30–40%). Since no ideal drug capable of curing all forms of epilepsy exists, the molecular identification of the genetic alterations responsible for each phenotype is the first step toward designing drugs capable of reversing the abnormality and thus developing personalized pharmacogenetic therapy.

Keywords Pharmacogenetics · Refractory Epilepsy (RE) · LADME system · ABC Transporters · CYPs · mTORopathies

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10.1 Introduction

Pharmacogenetics (PGt) is the scientific discipline that refers to a single genetic marker's effects on pharmacological therapeutic response. Pharmacogenomics (PGm) refers to the variability of genes detected in the genome that affect pharmacological therapeutics. An essential aspect of pharmacogenetics/pharmacogenomics involves not only mutations or polymorphisms that modify the function of implicated genes but also the nonmutated mechanisms of up-or-down-regulation of different genes critical for the modulation of drug effects.

Two scenarios in which PGt and PGm exert influence should be considered. First, several genes that govern the LADME (Liberation, Administration, Distribution, Metabolism, and Excretion) system and whose variants originated ancestrally in nutrigenomics are involved in drug biodistribution, biotransformation, and excretory systems. The LADME system is standard for virtually all drugs, although it differs among patients and drugs, affecting their pharmacokinetics (PK). Second, the genetic modifications that originate the mechanisms related to a specific disease or syndrome. Some of these molecular variants may be pharmacologically actionable to reverse their pathological action and are related to pharmacodynamics (PD).

Most, if not all, epileptic syndromes have a recommended pharmacological treatment with the influence of their respective LADME system (drugs-related genetic variants). However, only a select few also will have disease-related genetic variants requiring specific therapies directed against mutated molecules driving the disease (Mirzaev et al. 2019) (Fig. 10.1).

10.2 Pharmacogenetics of Antiseizure Medications and Their Relationship with Pharmacoresistant Epilepsy

Pharmacogenetics can influence drug efficacy pharmacokinetically and pharmacodynamically. Thus, this influence affects several features of therapeutic response, such as dosing, therapeutic sensitivity, and side effects, including the risk for hypersensitivity reactions. The hypothesis that candidate genes impact drug action was initially focused on different metabolizing subsystems involved in PK and assessed by PK studies.

At the beginning of the sixteenth century, Paracelsus stated that “only the dose makes a remedy poisonous,” perhaps establishing the “doses-response” relationship. The lack of knowledge of the biodistribution of drugs within the organism led for a long time to fatal iatrogenesis, in which the cause of death could not be distinguished between the disease itself or the drugs administered by physicians. Later, in the early 1970s, therapeutic drug monitoring (TDM) was introduced into medical practice to rationalize drug therapy by applying PK principles.

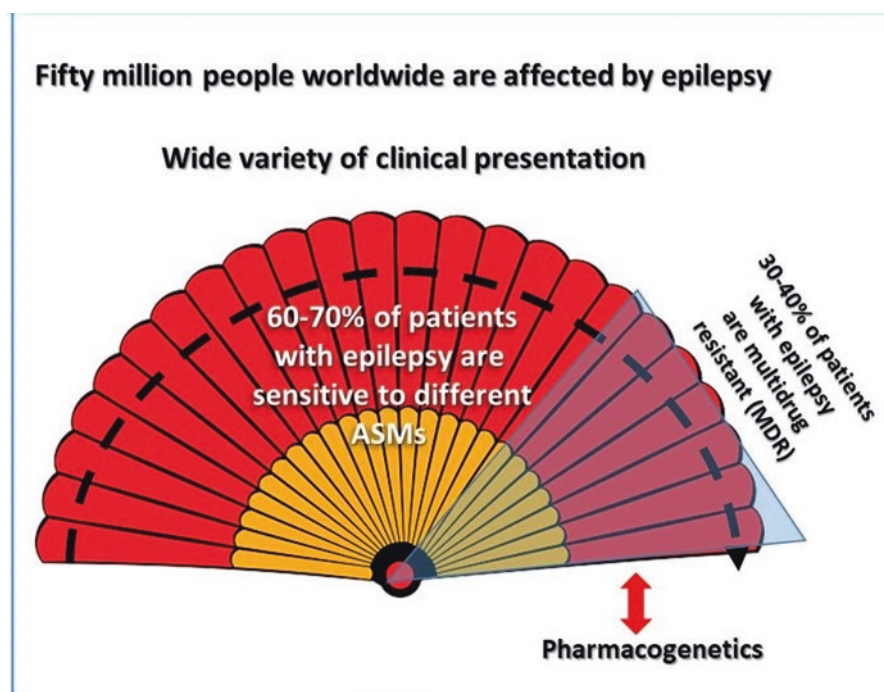


Fig. 10.1 Most patients with epilepsy achieve adequate seizure control using monotherapies with available antiseizure medications (ASMs). Only a fraction of patients remain uncontrolled and develop drug resistance. Pharmacogenetic tools should be explored to resolve the therapeutics for these patients

One of the first milestones in the pharmacokinetic analysis was the concept of clearance (Möller et al. 1928). From 1950 onwards, PK practice became a mandatory test not only for the development and subsequent approval of therapeutic drugs but also PK achieved an essential role in clinical practice, therapeutic follow-up of patients, and dose adjustment (Heath and Colburn 2000). Once the steady state of plasma concentrations within the corresponding therapeutic range is reached, the therapeutic response will be related to the administered dose. This allows an understanding of Paracelsus's concept: "only the dose makes a remedy poisonous."

As a consequence, these PK studies allowed individuals to be clinically classified as "fast," "rapid," or "extensive" metabolizers at one end of the spectrum, normal metabolizers, and "slow" or "poor" metabolizers at the other end of the population. Pharmacogenetics principles involving enzymes responsible for drug biotransformation are first analyzed in this field.

PK studies are often helpful for obtaining information on LADME events (Fig. 10.2).

Additionally, attention should be paid to age-related changes in these phenotypes. For example, newborns may be phenotypically "slow" or "poor"

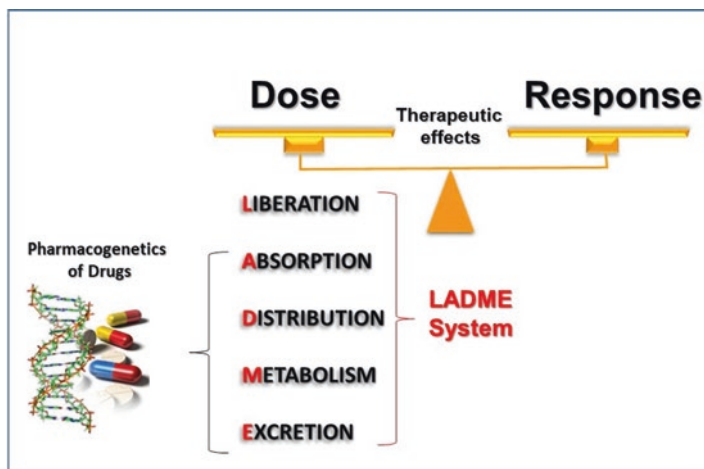


Fig. 10.2 In the face of insufficient, null, or adverse pharmacological response and no “drug quality” or patient compliance issues, PG mechanisms affecting the LADME system and drug pharmacokinetics should be considered

metabolizers due to the maturational developmental state of pathways, including glucuronidation and some of the cytochrome P450 (CYP) activities.

Except for drug liberation (related to pharmaceutical formulation), drug absorption, distribution, metabolism, and elimination are directly influenced by genetic variants governing each step.

10.2.1 *Pharmacogenetics of Drugs* (*Absorption-Biodistribution-Metabolism-Excretion*)

The physicochemical properties of drugs have long been considered the determining feature of their intestinal absorption, assumed to be a passive process. However, it is now known that carrier-mediated transport across membranes plays a fundamental role in drug and nutrient absorption (Anderle et al. 2004). Except for gabapentin and pregabalin, which are transported by large neutral amino acid carriers (L-system), ABC transporters (ABC-t) such as P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRP), and breast cancer-related protein (BCRP) expressed in enterocytes can limit drug absorption through their drug efflux activity in the intestinal lumen (Fig. 10.3).

Interestingly, the human *ABCB1* gene encoding the P-gp was shown to have several single-nucleotide polymorphisms (SNP) in Caucasians. One is located in exon 26 (C3435T) and correlates with changes in P-gp expression in the human intestinal tract with no amino acid sequence modifications (Hoffmeyer et al., 2000). In this study, individuals with the CC genotype had a twofold higher amount of P-gp

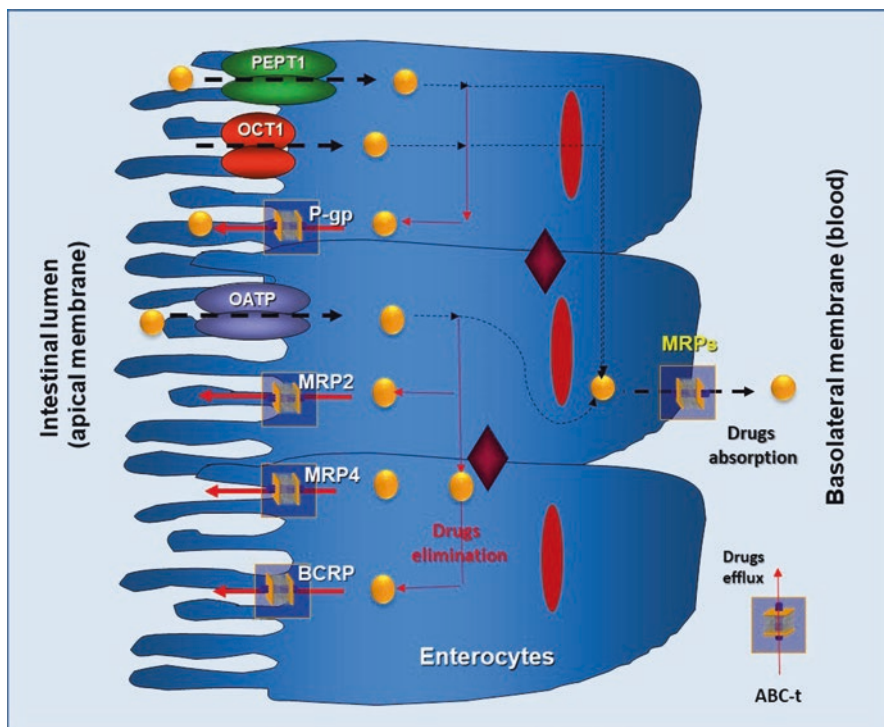


Fig. 10.3 The distribution of ABC transporters (ABC-t) at the intestinal level is polarized with a higher expression toward the intestinal lumen. Their increased expression secondary to different stimuli may reduce normal drug absorption. BCRP breast cancer-related protein; MRP multidrug resistance-associated proteins; OATP organic-anion-transporting polypeptides; OCT1 organic cation transporter-1; PEPT1 peptide transporter 1; P-gp P-glycoprotein

expressed in the duodenum than individuals with the TT genotype. After oral administration of equal doses of digoxin (Dgx) (a drug not metabolized and 100% transported by P-gp), CC cases showed significantly lower plasma concentrations than TT cases. Due to the inducible properties of P-gp, CC cases receiving rifampicin increased intestinal P-gp expression with a concomitant reduction in plasma Dgx concentration. In contrast, no changes in P-gp expression or Dgx concentration were observed in the TT group (Hoffmeyer et al. 2000).

These data suggest that this SNP of the *ABCB1* gene may not only be driving drug absorption in individuals with high levels of intestinal P-gp but could also be involved in increased expression at other critical sites related to brain access, such as the blood-brain barrier (BBB), or the hepatobiliary tract, modifying the PK and biodistribution of ASMs (and other therapeutics), with repercussions on their plasma levels. Therefore, it should be noted that some individuals in the population will be P-gp overexpressors, with an increased risk of developing a pharmacoresistant phenotype.

In their pioneering report, Tishler et al. documented that phenytoin (PHT) did not accumulate in DAOYAR2 cells expressing P-gp, encoded by the multidrug resistance 1 (*MDR-1/ABCB1*) gene. However, preincubating these cells with verapamil resulted in cellular accumulation of PHT to concentrations similar to those observed in control DAOY (*MDR-1* negative) cells (Tishler et al. 1995). Shortly after that, the persistence of low plasma PHT levels in a child with refractory epilepsy (RE) and high P-gp expression in dysplastic neurons in the epileptogenic brain region was documented for the first time (Lazarowski et al. 1999).

In this case, despite intravenous administration of 9 loading doses of PHT, plasma levels never exceeded values of 3–3.5 µg/ml. This report was followed by a second case with a similar characteristic phenotype plus an accelerated plasma clearance of PHT (Lazarowski et al. 2004a). In this latter case, administration of nimodipine (2 mg/kg) slowed plasma clearance of PHT (data not shown). Unfortunately, these cases were described before Hoffmeyer et al. discovery (Hoffmeyer et al. 2000), so the C3435T-SNP in exon 26 of the *ABCB1* gene was not investigated.

To confirm these observations, Ebid et al. detected the lowest PHT levels in individuals with CC genotype after PHT oral administration in healthy volunteers. Moreover, it was reported that epileptic patients with C3435C-SNP were more likely to present lower PHT levels (<10 µg/ml) than patients with T3435T-SNP (Ebid et al. 2007). Regarding the *ABCB1*-c.3435T>C variant in the Latin American context, it was reported that the number of patients with the TT genotype was considerably lower than the CC variant in a group of 202 Colombian patients with RE (Velasco-Parra et al. 2011).

In contrast, a meta-analysis demonstrated an opposite genotype relationship showing an increased risk of RE in cases with TT compared to the CC variant in Asians and Indians, suggesting that this opposite relationship compared to Caucasians might be an ethnic characteristic of Eastern populations (Chen et al. 2022).

This evidence indicates that differential expression of P-gp in the duodenum related to the different genotype of the C3435T SNP in the *ABCB1* gene has been well-documented and applies to other biological barriers where P-gp (and other ABC transporters) is constitutively expressed, such as the bile canaliculus, kidneys, and BBB (Cordon-Cardo et al. 1990).

Generally, CYPs and glucuronidation (GLN) systems (Fig. 10.4a, b) metabolize drugs that ABC-t then eliminates (Fig. 10.4c) via bile. However, certain compounds are not metabolized and are directly excreted by ABC-t (Fig. 10.4a).

Additionally, CYP3A4 and CYP2C9/19 can metabolize and degrade orally administered drugs, decreasing the final quantity of drugs to be absorbed. Moreover, these enzymes can be induced by some commonly administered ASMs, such as carbamazepine. The so-called transporter hypothesis regarding the cause of RE is based on overexpression of ABC-t in the BBB, limiting the access of ASMs to the brain, and the so-called pharmacokinetic hypothesis considers a sum of effects produced by elevated activity of ABC-t in excretory organs, as well as increased activity of ASMs metabolic systems (mainly CYP enzymes), affecting the PK and

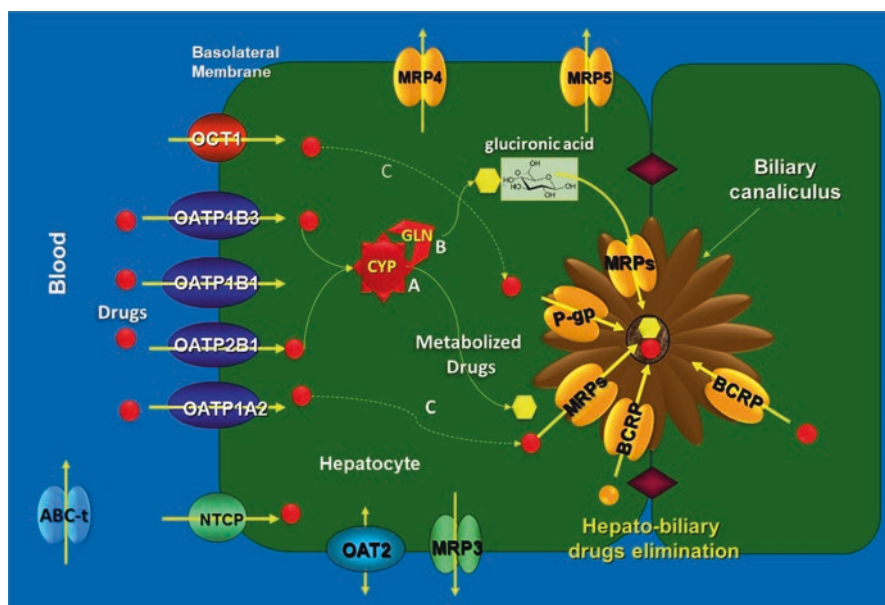


Fig. 10.4 Several transporters participate in the delivery of drugs to the liver and at the level of the bile canaliculi, mainly ABC-t, which concentrate their excretory activity to eliminate both metabolites and intact drugs. (a) The CYP system (phase I of metabolism) is involved in the metabolic degradation of drugs. (b) Metabolized drugs will be conjugated in phase II of glucuronidation (GLN) and then excreted mainly by hepatic P-gp encoded by the *MDR-3* gene, BCRP, and MRPs. (c) Some drugs can be directly excreted without metabolic changes. NTCP: Na⁺-taurocholate co-transporting polypeptide; OCT1: organic cation transporter-1; OATP organic-anion-transporting polypeptides; OAT2: organic anion transporter 2

plasma levels of ASMs (Tang et al. 2017). In a recent critical review, the authors postulated that the ABC-t plays a role in RE phenotype in peripheral organs and centrally (brain) (Czornyj et al. 2022).

Recently, single nucleotide polymorphisms (SNP) of CYP2D6, CYP2C9, CYP2C19, and CYP3A4 have been studied in a rigorously selected Mexican population of pediatric patients with drug-resistant epilepsy. In this study (23 patients with drug-resistant epilepsy and seven patients with good response), the relevant SNPs with pharmacogenomics relationship were CYP2D6*2 (rs16947) for decreased activity and CYP2D6*4 (rs1065852), CYP2C19*2 (rs4244285), and CYP3A4*1B (rs2740574) for association with poor metabolizer type. The most substantial risk factors were found in the AA genotype and the rs3892097 SNP allele of the CYP2D6 gene, followed by the A and T alleles of the rs2740574 and rs2687116 SNPs, respectively, of CYP3A4. The most significant concomitance was between the AA homozygous genotype of rs3892097 and the AA genotype of rs2740574, with 78.3% in drug-resistant epilepsy patients versus 14.3% in controls. These results strongly suggest the critical role of the CYP3A4*1B allelic variant as a risk factor for developing drug resistance. CYP2D6, CYP2C19 SNPs, and haplotypes may affect the response to antiepileptic drugs (López-García et al. 2017).

P-gp without enzymatic transformation can excrete some drugs, such as Dgx, and radio-compounds, such as ^{99m}Tc -SESTAMIBI. This property allows measuring the traceability of the radiopharmaceutical, not only to determine its reduced passage through the BBB, associated with the C3435C and G2677G ABCB1 genotypes that also correlate with ASMs resistance (Jensen et al. 2006; Löscher 2007) but also accelerated hepatobiliary excretion in cases of surgically treated RE patients with P-gp overexpression in neurons of their epileptogenic brain area (Vazquez et al. 2004; Czornyj et al. 2022). An interesting mechanism to consider is the ability to induce an increase in the expression of these systems. Physiologically they are synchronized in their metabolizing and excretory activities. In addition, the functional interactions between metabolizing enzymes and drug transporters is a critical issue in pharmacokinetic considerations, where the sum of different genetic haplotypes of these systems can conjugate to increase or decrease the ability to metabolize/excrete drugs. Löscher (2007) published a comprehensive review of genetic variants related to ASMs absorption, biodistribution, metabolism, and excretion (Fig. 10.4) (Löscher 2007).

CYP3A and P-gp share the same regulatory mechanisms, such as the pregnane X receptor (PXR). There is significant genetic variability of CYP3A (the predominant CYP family in the human liver), P-gp, and PXR. Their expression and activity are sensitive and dependent on co-administered drugs, food, age, hormonal status, and disease. Comprehensive information on the interaction between CYP and P-gp has been extensively reviewed (Christians et al. 2005).

The study of the combination of some ABC-t and CYPs polymorphisms and their relationship with the RE phenotype in Mexican patients with epilepsy reported that of six ABCC2-associated SNPs found in the study population, only rs3740066 (TT) and 66744T> A (TG) were observed in the RE cases. The highest risk factor in the *ABCB1* gene was identified as the TA genotype of rs2032582, whereas for the *ABCC2* gene, the highest risk factor was the T allele of rs3740066. Screening for SNPs in *ABCB1* and *ABCC2* indicated that these Mexican patients with epilepsy showed frequently reported *ABCC1* polymorphisms. However, in subjects with a higher risk factor for drug resistance, novel nucleotide changes were found in the *ABCC2* gene (Escalante-Santiago et al. 2014). All these genetic variants are directly related to dose-response balance, where dose adjustment and transporter inhibition may help to better control seizures in RE patients. Persistent low levels of ASMs should be analyzed under this broad spectrum of genetic variants and not only assume patient compliance issues.

Regarding drug inducers in epilepsy, the literature suggests that drug-drug interactions play a role in the PK of ASMs. In a recent review, CYP isoenzymes CYP2B6 and CYP3A4 were found to be very sensitive to induction by ASMs (López-García et al. 2014; De Leon 2015; Guevara et al. 2017) (Fig. 10.4).

Similarly, it was experimentally demonstrated that overexpression of efflux transporters could be mediated by inducer agents, such as PHT, in a local concentration-dependent manner, reversible once the substance is cleared from the body. Recovery of basal P-gp expression could allow the design of dosing schemes that optimize anticonvulsant therapy (Alvariza et al. 2014).

10.2.2 Pharmacogenetics of Cannabidiol

Although studies indicate that the efficacy of phytocannabinoids as antiseizure therapy is contradictory, cannabis compounds have gained significant interest due to their favorable effects on seizure control in patients with drug-resistant epilepsy. Particular interest has been placed on children with severe (catastrophic) epileptic syndromes such as Dravet (pathogenic variants in the sodium channel gene *SCN1A*) or Lennox-Gastaut syndromes. Clinical evidence supports that pediatric or adult patients with refractory epileptic disorders can achieve significant improvement with cannabidiol (CBD) administration (Rocha et al. 2020).

Few studies have investigated the pharmacogenetic characteristics of CBD concerning the treatment of RE. In this regard, using the Affymetrix Drug Metabolizing Enzymes and Transporters plus array, the association between genetic variants with good CBD response ($\geq 50\%$ seizure reduction) or poor tolerability (diarrhea, sedation, and abnormal liver function) was evaluated in patients with RE enrolled in the Expanded Access Program (EAP) to CBD at the University of Alabama at Birmingham. This study assessed a broad spectrum of genes associated with CBD response, such as phase I/II metabolism genes, SLC family transporters, and ABC family transporters, among others. Patients with AOX1 rs6729738 CC (aldehyde oxidase) or ABP1 rs12539 (diamine oxidase) were more likely to respond well. Conversely, patients with SLC15A1 rs1339067 TT were less likely to respond. Additionally, ABCC5 rs3749442 was associated with a lower likelihood of response and abnormal liver function tests (LFT). The rs3749442A allele, associated with CBD response, sedation, and abnormal LFTs, was also associated with lower expression of HTR3E (serotonin 5-HT_{3E} receptor) in the hippocampus. This study demonstrated that genes implicated in CBD response are also involved in fundamental biologic processes. It may provide new insights into the mechanisms through which CBD exerts its therapeutic effects in patients with RE and the potential genetic underpinnings of treatment resistance (Davis et al. 2021).

Furthermore, independent reports demonstrated that CBD exerts an inhibitory effect on the most abundant CYP isoforms (70%) (Fig. 10.4), such as CYP3A (Yamaori et al. 2011), CYP2D6, CYP2C9, and CYP2B6 (Nasrin et al. 2021), or CYP2C19 (Jiang et al. 2013), and CYP2C19 and CYP2C9 (Doohan et al. 2021), suggesting that these inhibitions could affect the normal metabolism of ASMs. In the same line of reasoning, our group and other authors demonstrated that CBD inhibits P-gp efflux activity, with two specific binding sites of CBD to the P-gp molecule (Auzmendi et al. 2020) or inhibition of both function and expression of ABC-t (Holland et al. 2006, 2007; Zhu et al. 2006; Feinshtein et al. 2013; Brzozowska et al. 2016). All these mechanisms could improve the biodistribution of ASMs, their access to the brain, and reduce their metabolism and excretion. Therefore, it would be feasible to expect increased brain concentrations of ASMs when combined with CBD (Fig. 10.5).

In pediatric patients with RE, the administration of clobazam (CLB) plus CBD resulted in increased plasma levels of both CLB and N-desmethyclobazam

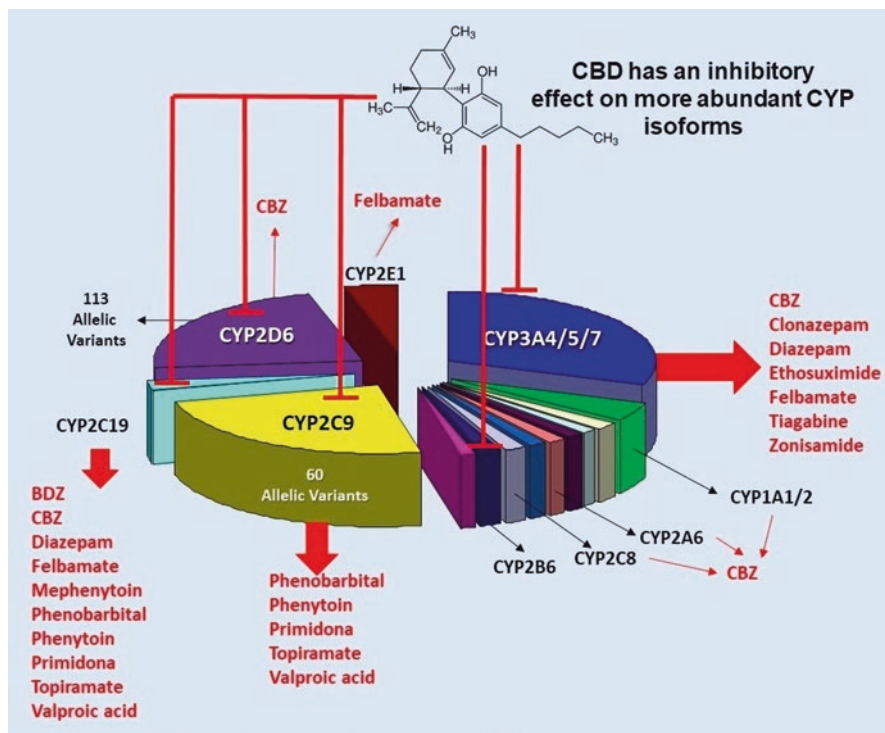


Fig. 10.5 Main CYP isoforms responsible for the metabolism of the more frequently used ASMs (→) and the inhibitory effects of CBD on more abundant CYPs isoforms (⊥)

(norclobazam; nCLB), the active metabolite of CLB. This effect was attributed to the fact that the metabolism of CLB to nCLB involves CYP3A4 as the primary enzyme, and to a lesser extent, CYP2C19 is also involved. Both CYP isoforms are inhibited by CBD (Geffrey et al. 2015). In another study in 39 adults and 42 children, CBD was started at a dose of 5 mg/kg/day and increased every 2 weeks by 5 mg/kg/day up to a maximum dose of 50 mg/kg/day. Baseline serum ASM levels were determined before CBD treatment and at most visits during the trial. Significant changes in serum levels of clobazam, rufinamide, topiramate, zonisamide, and eslicarbazepine were observed. Abnormal LFT results were found in participants taking concomitant valproate. This study emphasizes the importance of monitoring serum ASM levels and LFTs during CBD treatment (Gaston et al. 2017).

Carbamazepine (CBZ) can be metabolized by several CYP isoforms (Fig. 10.5), which CBD can inhibit. It has recently been described that patients with HLA immunophenotypes HLA-B*15:02, HLA-B*15:11, HLA-B*15:21, HLA-B*38:02, HLA-B*40:01, HLA-B*46:01, HLA-B*58:01, HLA-A*24:02, and HLA-A*31:01 may develop severe CBZ-induced cutaneous adverse drug reactions (cADRs) or Stevens-Johnson syndrome (Biswas et al. 2022). In these cases, the combined administration of CBZ and CBD should be avoided.

A recent report found that patients with RE who received brivaracetam (BRV) plus CBD showed a significant increase (95% to 280%) in plasma BRV levels. One possible mechanism contributing, at least partially, to these increased BRV levels is the inhibition of CYP2C19 by CBD (Klotz et al. 2019).

An experimental PK study in mice treated with gabapentin plus CBD demonstrated that both serum and brain concentrations of gabapentin increased by ~30% and 40%, respectively, indicating a pharmacokinetic interaction between both drugs. A similar increase was reported for topiramate (~30%) and oxcarbazepine (~20%). Moreover, adjuvant CBD administration has been observed to increase brain concentrations of tiagabine (~50%). Interestingly, lacosamide and CBD could mutually increase penetration across the BBB because CBD caused a 35% increase in the brain concentration of lacosamide, and lacosamide increased the brain levels of CBD by ~70% (Socała et al. 2019).

Overall, this evidence strongly supports the central role of CBD as a potential sensitizer to ASMs treatment in patients with RE. In addition, CBD was recently reported to reduce short- and long-term experimental traumatic brain injury (TBI)-induced excitotoxicity and facilitate functional recovery, suggesting CBD administration as an opportunity in the preventive treatment of the development of epilepsies secondary to brain trauma (Santiago-Castañeda et al. 2022).

10.2.3 Pharmacogenetics of Antiepileptic Drugs Adverse Drug Reactions

Evidence has shown that variant polymorphism of *CYP2C9* and *CYP2C19* genes can lead to significant differences in serum ASM concentrations (López-García et al. 2014). *CYP2C9* is responsible for about 90% of PHT metabolism and plays a rate-determinant role on PHT metabolism. In addition to the wild-type *CYP2C9**1 protein, at least five allelic variants produce allozymes with reduced or low metabolic activity. Individuals carrying *CYP2C9* alleles encoding variant enzymes (allozymes *CYP2C9**2 (rs1799853) and *CYP2C9**3 (rs1057910(C)) are the best documented), have an reduced enzymatic activity, and metabolize PHT at a considerably slower rate compared to individuals homozygous for the wild-type allele (*CYP2C9**1; rs1057910(A)). Therefore, they have an increased risk of developing concentration-dependent neurotoxicity. The maximum PHT dose reported in a series of individuals with epilepsy was about 50 mg less per *CYP2C9**3 allele (Tate et al. 2005).

A cluster of 16 SNPs in *CYP2C* genes was recently discovered regarding severe cutaneous adverse reactions. The missense variant rs1057910 (*CYP2C9**3) was associated with severe cutaneous adverse reactions of PHT (Chung et al. 2014).

Similarly, an immunogenetic association with adverse drug reactions (ADRs) to ASMs has been well established between the HLA-B*1502 allele and severe cutaneous ADRs to CBZ treatment in the Han Chinese population (Depontd 2008).

More specific results were recently reported showing that HLA-B*15:11 is a potential risk factor for CBZ-induced severe cutaneous adverse drug reactions (SCARs) in HLA-B*15:02 negative Chinese patients. Further screening for HLA-B*15:11 status is recommended in those HLA-B*15:02 negative patients to avoid unwanted SCARs (Wong et al. 2022). Similar results were observed in Brazilian patients (Perelló et al. 2022). Another study reported that decreased CYP2C9 allele function was highly predictive of vestibular-cerebellar ADRs to phenytoin (Calderon-Ospina et al. 2020).

Hyperhomocysteinemia—a clinical condition commonly related to folate and vitamin B₁₂ deficiency—may be a risk factor associated with long-term treatment with ASMs, as ASMs interfere with folic acid (FA) and homocysteine (Hcy) metabolism. Munisamy et al. investigated the MTHFR C677T polymorphism in epileptic patients receiving ASMs as monotherapy (PHT, CBZ, and sodium valproate). A significant increase in Hcy levels was detected in epileptic patients carrying the MTHFR C677T polymorphism (Munisamy et al. 2015). These results suggest that these patients could be favored with vitamin B₁₂ supplementation, especially in developing countries with poor nutritional scores.

10.3 Gene Mutations Related to Epilepsy and Potential Pharmacogenetic Therapeutic Targets

Epilepsy treatment relies on a wide range of ASMs with different mechanisms of action. However, some ASMs overlap the same therapeutic targets or function with identical or equivalent mechanisms of action (Fig. 10.6).

The heterogeneity of the various epileptic syndromes and the interindividual differences in response to ASMs mean that treatment of this condition remains a challenging task. Despite advances in the design of new ASMs, paradoxically, it has not been possible to reduce the constant proportion (30–40%) of patients who remain without adequate seizure control and develop the RE phenotype.

It should be mentioned that several mutated genes are present in RE. Although most of these mutations are found in the same gene (ion channels, receptors, or metabolic intermediaries), the clinical phenotypes are very different.

This broad spectrum of mutations is associated with diverse clinical presentations and therapeutic response phenotypes (responders or nonresponders).

More than 900 epilepsy-related genes have been described in this list, of which 84 genes causing epilepsies or syndromes with epilepsy as a primary symptom have been added (e.g., mutations in *SCN1A* cause epilepsies with febrile seizures +), and another 73 genes were classified as neurodevelopment-associated genes e.g., mutations in *TSC1* and *TSC2* genes that cause tuberous sclerosis or those genes associated with brain-development malformations, all related with epilepsy (Wang et al. 2017). It is estimated that more than half of epilepsies have a genetic basis (Pal et al. 2010) (Fig. 10.7). Interestingly, the same gene can have different mutations

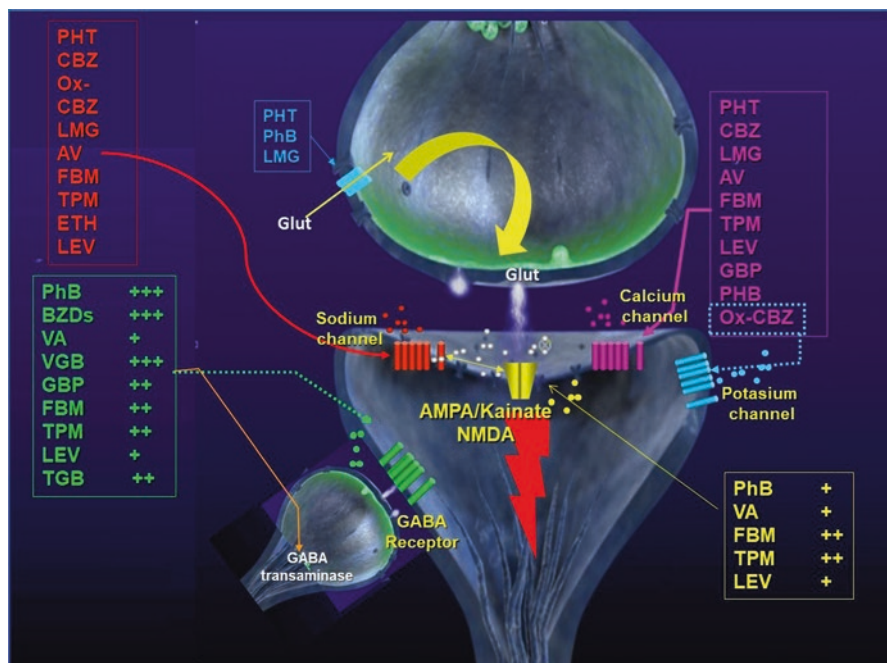


Fig. 10.6 Spectrum of old and new ASMs, many of which act on the same targets or mechanisms. BZD: benzodiazepines; CBZ: carbamazepine; ETH: ethosuximide; FBM: felbamate; GBP: gabapentin; LEV: levetiracetam; LMG: lamotrigine; Ox-CBZ: oxcarbazepine; PhB: phenobarbital; PHT: phenytoin; TGB: tiagabine; TPM: topiramate; VA: valproic acid; VGB: vigabatrin

associated with different clinical phenotypes. Therefore, saying that a patient has a mutation in a specific gene is not enough to identify the related type of epilepsy, so the particular mutation must be identified.

10.3.1 Mutations in Neurotransmitter Receptors

The GABA_A receptor is a heteromer composed of five subunits, most commonly two α , two β , and one γ ($\alpha 2\beta 2\gamma$), and many subtypes such as α_{1-6} , β_{1-3} , and γ_{1-3} in each subunit. Each subunit has a specific binding affinity to GABA (β subunit) or benzodiazepines (BDZ) in the γ subunit. GABA_A receptor subunits are translated as a precursor protein whose signal sequence (left end) is removed, leaving a mature protein consisting of a large extracellular domain at the N-terminus, four (M1–M4) transmembrane domains (TMD), and a large cytoplasmic domain connecting TMD M3–M4 (Fig. 10.8).

Furthermore, mutations in the NMDA receptor subunits have been reported in several epileptic syndromes, intellectual disability, and developmental delay

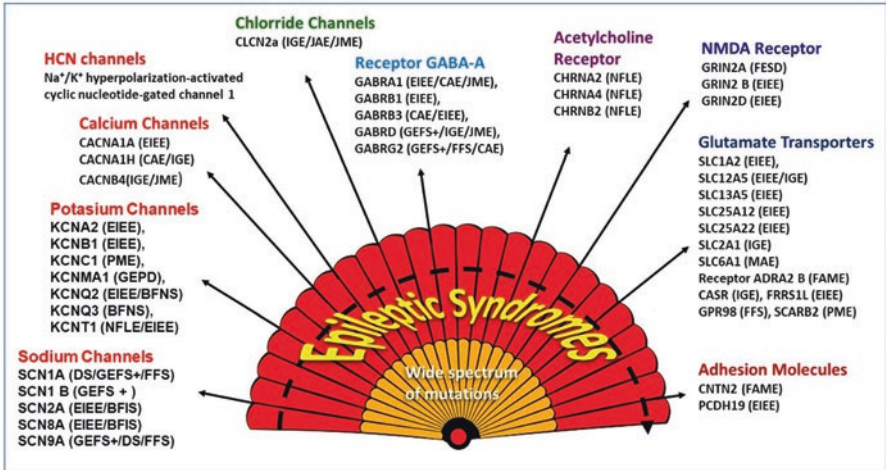


Fig. 10.7 Primary epilepsy genes. Epilepsies can result from a broad spectrum of primary and secondary genetic abnormalities to well-defined structural or metabolic disorders, of which some also have genetic causes (Lazarowski and Czornyj 2013; Wang et al. 2017)

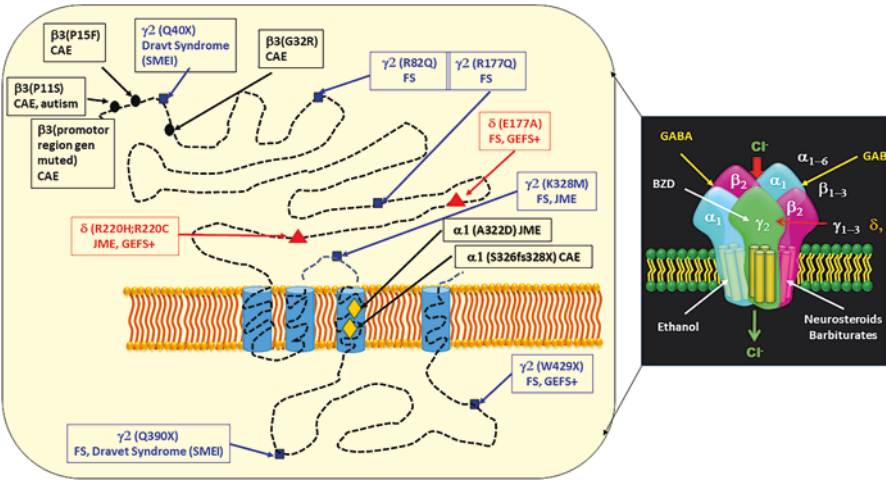


Fig. 10.8 GABA_A receptor and its mutations. Schematic representation of the typical structure of GABA_A receptor subunits, indicating specific mutation points in their appropriate protein domain within their respective subunit. These mutations are associated with genetic epilepsy syndromes (CAE: childhood absence epilepsy; DS: Dravet syndrome; FS: pure febrile seizures; GEFS+: generalized epilepsy with febrile seizures plus; JME: juvenile myoclonic epilepsy; SMEI: severe myoclonic epilepsy in infancy). (Adapted from Macdonald et al. 2010)

(Burnashev and Szepietowski 2015) (Figs. 10.9 and 10.10). Mutations in the glutamate ionotropic receptor NMDA type subunit 2A (GRIN2A or GluN2A) gene causes a spectrum of childhood-onset epilepsy syndromes, including

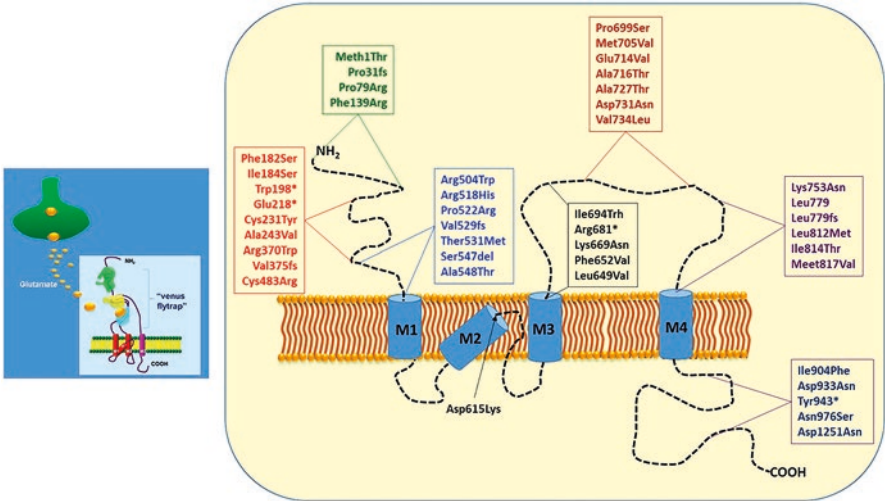


Fig. 10.9 Localization of various mutations found in NMDARs GluN2A subunits. Numbers indicate amino acid positions

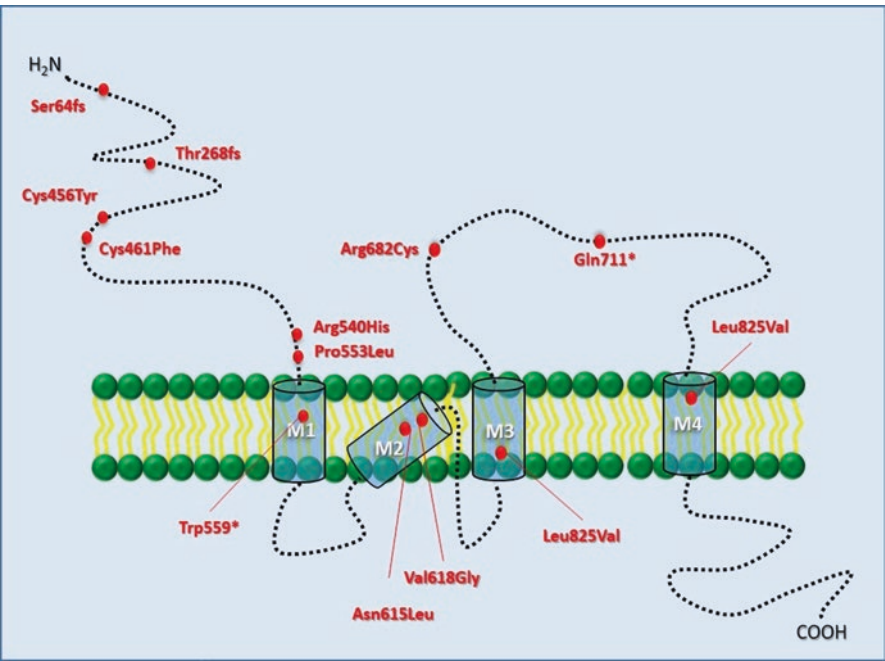


Fig. 10.10 Representation of mutations in the NMDAR GluN2B subunit

Landau–Kleffner syndrome (LKS), epileptic encephalopathy with continuous spike-and-wave during sleep (ECSWS), childhood epilepsy with centrottemporal spikes (CECTS), atypical childhood epilepsy with centrottemporal spikes (ACECTS), autosomal dominant rolandic epilepsy with speech dyspraxia (ADRES), and early-infantile onset epileptic encephalopathy, developmental delays, and speech and language disorders (Balestrini and Sisodiya 2018). In this complex variability of syndromes, Pierson et al. documented that the use of memantine, an NMDA receptor antagonist, inhibited the increased activity of de novo GRIN2A missense mutation (c.2434C>A; p.L812M), with a significant decrease in seizure frequency and improvement in interictal EEG recordings (Pierson et al. 2014).

One of the more complex epileptic syndromes is perhaps Dravet syndrome (DS), a severe and catastrophic epileptic encephalopathy. DS can arise from different mutations in the same gene of the voltage-gated sodium channel $\alpha 1$ subunit gene (*SCN1A*), which is the most frequent cause of DS. More than 1800 mutations in the *SCN1A* gene have been identified, and 80% cause DS (Ding et al. 2021). *SCN1A* dysfunction may be associated with various phenotypes ranging from severely affected patients with DS to febrile seizures plus (FSGS+), genetic epilepsy with much less severity than DS.

However, DS or DS-like phenotypes can also arise from mutations in other genes such as protocadherin 19 (*PCDH19*), other Na^+ channels (*SCN2A*, *SCN8A*, and *SCN1B*), GABA receptors (*GABRA1*, *GABRG2*, and *GABRB3*), hyperpolarization-activated and cyclic nucleotide-gated potassium channel 1 (*HCN1*), syntaxin-binding protein 1 (*STXBP1*), chromodomain helicase DNA-binding protein 2 (*CHD2*), and potassium voltage-gated channel subfamily A member 2 (*KCNA2*) (Steel et al. 2017). The wide variety of genetic alterations causing DS has not yet allowed the development of specific targeted therapeutics related to the molecular alteration in each case. The best currently recommended DS therapeutics is the combined administration of stiripentol, valproate, and clobazam, with still controversial results (Balestrini and Sisodiya 2017).

10.3.2 Drug-Responsive Epileptic Syndromes Associated with Specific Mutations

Pyridoxine (Vitamin B₆)-Dependent Epilepsy

Several genetic alterations cause pyridoxine (vitamin B₆)-dependent epilepsy (PDE). These alterations impair access to the brain and proper or adequate use of pyridoxal 5' phosphate in enzymatic steps directly related to epileptic phenomena and oxidative stress. Bi-allelic mutations have been identified in the *ALDH7A1* gene [Aminoacidic Semialdehyde (α -AASA) Dehydrogenase Deficiency syndrome], which encodes antiquitin (ATQ). ATQ deficiency is the primary cause of PDE, characterized by an early-onset epileptic encephalopathy responsive to large doses of pyridoxine (Stockler et al. 2011). ATQ plays an aldehyde dehydrogenase role in

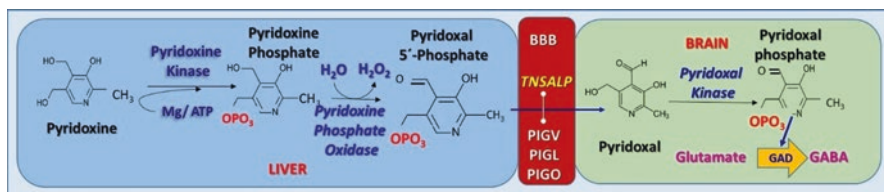


Fig. 10.11 Pyridoxine is phosphorylated to pyridoxine phosphate (a water soluble compound) in the liver to be used in the peripheral system. However, it cannot cross the BBB, so it must be dephosphorylated by the Tissue Nonspecific Alkaline Phosphatase (TNSALP). Pyridoxal (a fat-soluble compound) will cross the BBB and, once in the brain, is again phosphorylated by pyridoxal kinase. Therefore (PK), so intracerebral pyridoxal phosphate will be the cofactor for Glutamate Decarboxylase activity to produce GABA

lysine metabolism (degradation). This syndrome is characterized by seizures that are not well controlled with antiseizure medications but are clinically and electrographically responsive to large daily pyridoxine (vitamin B₆) supplements. The phenotypic spectrum of this syndrome ranges from classic to atypical PDE-ALDH7A1. Intellectual disability is frequent, particularly in classic PDE-ALDH7A1. Although seizure control is observed in every case following B₆ therapeutics, at least 75% of individuals have an intellectual disability and developmental delay (Coughlin et al. 2019). Antiquitin deficiency causes seizures because the accumulation of Δ^1 -piperidine-6-carboxylate (P6C) condenses with pyridoxal 5'-phosphate (PLP) and inactivates this enzyme cofactor, essential for the normal metabolism of neurotransmitters. Similar effects can be observed in other genetic epilepsies with mutations in the pyridox(am)ine 5'-phosphate oxidase (*PNPO*) gene. Some cases are best treated with pyridoxal 5'-phosphate (Mills et al. 2014).

Furthermore, mutations in tissue nonspecific alkaline phosphatase (TNSALP) will prevent access of pyridoxine to the central nervous system (CNS) (Fig. 10.11). In addition, mutations in the *PIGV*, *PIGL*, and *PIGO* genes (the GPI anchors) will prevent TNSALP localization to the BBB, inducing a similar brain deprivation of vitamin B₆. In these cases, treatment with pyridoxine can compensate for this deficiency (Fig. 10.11).

Mutations have been described at the level of PLP metabolism, such as PNPO (Pyridoxine Phosphate Oxidase), TNSALP (Tissue Nonspecific Alkaline Phosphatase), PIGV, PIGL or PIGO (GPI- anchors), PROSC (Proline Synthase Cotranscribed, or Pyridoxal Phosphate Homeostasis Protein), PDXK (Pyridoxal Kinase), and mutations causing inactivation of PLP, such as ALDH7A1 (α -Aminoacidic Semialdehyde Dehydrogenase), MOCS2 (Molybdenum Cofactor Synthesis 2, involved in the oxidation of sulfite to sulfate), and ALDH4A1 (P5C dehydrogenase, metabolism of proline).

In all these cases, recommended high doses of pyridoxine should control long-term neurotoxic effects to prevent the development of treatment-associated polyneuropathies (Kulkantrakorn 2014; Hassel et al. 2019).

Folinic Acid Responsive Seizures

A long list of progressive white-matter abnormalities and seizures in the neonatal period has been reported with a broad spectrum of differential diagnoses, including folinic acid-responsive seizures (FARS), which share the same cerebrospinal fluid monoamine metabolite profile with pyridoxine-dependent epilepsy (detected by high-performance liquid chromatography and electrochemistry). In these cases, seizure disorders are refractory to standard ASMs. However, according to some studies, seizures could be controlled within 24 hours after starting folinic acid administration (Frye et al. 2003; Gospe 2022).

Folinic acid, the activated form of folic acid, is a cofactor involved in one-carbon transfer reactions required for methylation and synthesizing purines, such as methionine and thymidylate, where vitamins B₆, B₁₂, and folic acid are cofactors in these pathways. Serine methyltransferase (SHMT), together with the pyridoxal phosphate (PLP) cofactor, transfers a methyl group on tetrahydrofolate (THF) to produce 5,10-MTHF allowing the recovery and reuse of homocysteine for the synthesis of glutathione (antioxidant system), also dependent on vitamin B₆ (Fig. 10.12).

Since the first description of FARS in 1995 by Hyland et al. (1995) in two infants (one male and one female), only a few additional cases have been reported. In this convulsive syndrome, a pattern of progressive cortical atrophy and white-matter changes found on MRI is observed, features also commonly observed in mitochondrial defects. In these cases, an energetic mitochondrial defect was suggested to explain the potential adverse effect of phenobarbital (PhB) use in these patients because PhB can inhibit glucose transport into the cerebrospinal fluid (Klepper and Voit 2002).

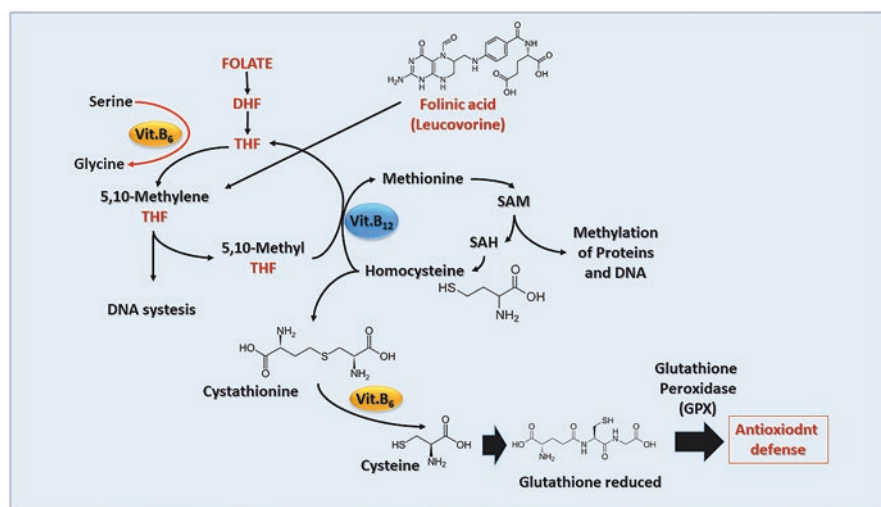


Fig. 10.12 Folinic acid (leucovorin) acts by rescuing 5-methyl-THF and recovering the normal homocysteine–methionine balance with vitamin B₆

Supportive laboratory findings in both syndromes

- Elevated plasma and urinary concentrations of alpha-aminoadipic semialdehyde (α -AASA), a nonspecific biomarker, could also be elevated in individuals with molybdenum cofactor deficiency and isolated sulfate oxidase deficiency.
- Elevated plasma and cerebrospinal fluid pipecolic acid concentrations might normalize after many years of treatment.

10.3.3 Glucose Type 1 Transporter Deficiency

Another example of a genetic mutation related to epilepsy with specific therapeutics is glucose type I transporter (GLUT-1) mutation due to the autosomal dominant inheritance of *SLC2A1* gene mutations (Suls et al. 2008).

These mutations are associated with impaired glucose transport across the BBB, leading to drug-resistant epilepsy, intellectual disability, and movement disorders. Because ketones can replace the lack of energy due to intracerebral glucose deficiency, the ketogenic diet (KD) is the recommended therapeutic that achieves, in these cases, seizure control but controversial results in neurodevelopment (De Giorgis and Veggiotti 2013).

GLUT-1 deficiency may be associated with movement disorders, such as paroxysmal exercise-induced dyskinesia (PED), or complex motor disorders with dystonia and spasticity, ataxia, and seizure episodes that may occur separately or in combination with movement disorders. Intractable infantile seizures, complex motor disorders, and intellectual disability are the clinical presentation of the most severely affected individuals with classic GLUT1 encephalopathy (Mullen et al. 2010). Since decreased glucose transport into the brain leads to seizures and cognitive dysfunction, the ketogenic diet is the treatment of choice in these cases (Almuqbil et al. 2015).

10.3.4 Pharmacogenetics of Epileptic mTORopathies

Several malformations of cortical development (MCD) are associated with epileptic syndromes with a drug-resistant phenotype. This group of neurodevelopmental disorders includes tuberous sclerosis complex (TSC), focal cortical dysplasia type II (FCDII), polyhydramnios, megalencephaly, and symptomatic epilepsy (PMSE) syndrome, and hemimegalencephaly (HME), among others. In these disorders, hyperactivation of the mammalian target of rapamycin (mTOR) signaling is common to different gene mutations. Interestingly, in MCD and brain tumors, overexpression of ABC transporters has been associated with the pharmacoresistant phenotype (Czornyj and Lazarowski 2014).

In the last decade, increasing evidence demonstrated that hyperactivation of the mTOR pathway is a hallmark of MCD, such as focal cortical dysplasia (FCD) or

hemimegalencephaly. Activating somatic mutations in the *MTOR* gene are the most frequent mutations found in FCD brain specimens (Marsan and Baulac 2018).

Tuberous Sclerosis Complex

The first syndrome in which genetic abnormalities related to the mTOR pathway were documented was TSC, in which mutations in the tumor suppressor genes *TSC1* and *TSC2* occur in most patients, leading to hyperactivation of the mTOR signaling pathway and consequent multisystem abnormalities.

Mutations in either of these two tumor suppressor genes, such as hamartin (*TSC1* gene) or tuberin (*TSC2* gene), lead to loss of control over the mTOR signaling pathway, resulting in abnormalities in numerous cellular processes (Crino 2015).

One of the most commonly affected organ systems in TSC is the CNS (in 85–90% of children and adolescents). Disabling neurological manifestations identified include epilepsy (66–93% of patients with TSC), subependymal nodules (SENs in 90–100%), subependymal giant cell astrocytomas (SEGAs in 5–20%), mental retardation (44–64%), and infantile spasm (45%) (Curatolo et al. 2008). Some of these syndromes are drug-resistant epilepsies (Hallett et al. 2011). Overexpression of ABC transporters has been reported in both epileptic cases of TSC with cortical tubers and SEGA (Lazarowski et al. 1999, 2004b, 2006, 2014).

Polyhydramnios, Megalencephaly, and Symptomatic Epilepsy Syndrome

Polyhydramnios, megalencephaly, and symptomatic epilepsy syndrome (PMSE) is a sporadic and severe intractable infantile-onset epilepsy with neurocognitive delay, macrocephaly, and craniofacial dysmorphism (Puffenberger et al. 2007).

The molecular abnormality is a 7 kb homozygous deletion of exons 9–13 of the *LYK5/STRADA* gene, which encodes the STRADA pseudo kinase, an upstream inhibitor of the mammalian target of rapamycin complex 1 (mTORC1). The recessive disease, caused by a small deletion of the *LYK5/STRADA* gene, is found exclusively in the Old Order Mennonites. Recessive disorders are regularly observed in these communities, including homozygous *CNTNAP2* mutations in symptomatic recessive focal epilepsy in the Amish (Strauss et al. 2006).

STRADA encodes for the TE20-related kinase adaptor alpha. Somatic mutations in STRADA may decrease stimulation with reduced *TSC1/TSC2* activity and consequently increased mTOR activation (Fig. 10.13). Mutations in STRADA cause PMSE. Parker et al. demonstrated that sirolimus (rapamycin) treatment could dramatically reduce seizure frequency in PMSE (Parker et al. 2013).

In a 4-year-old Indian male with global developmental delay, a history of growth retardation, infantile spasms, repetitive behaviors, hypotonia, low muscle mass, and marked joint laxity. Also, dysmorphic facial features, including a high forehead, long face, arched eyebrows, small chin, wide mouth, and tent-like upper lip, were observed, so the whole exome was sequenced (WES). A homozygous single

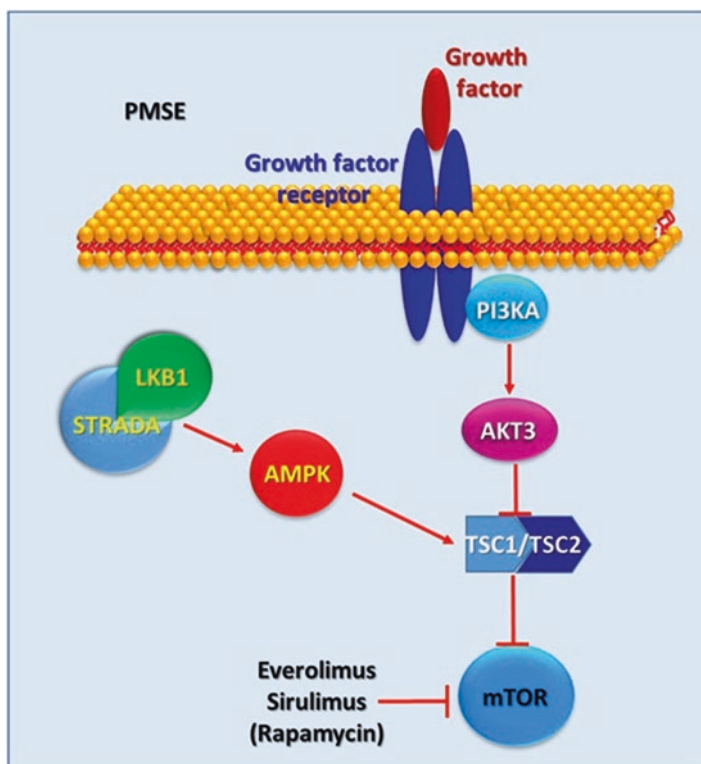


Fig. 10.13 LKB1/STRADA pathway interacting with TSC1/TSC2 complex and mTOR activity control

nucleotide duplication, c.842dupA (p.D281fs), was identified in exon 10 of the *STRADA* gene. Sanger sequencing confirmed the mutation in this individual and identified both parents as carriers. In light of the molecular findings, the patient's clinical phenotype was considered to fit well with PMSE. A homozygous point mutation in *STRADA* causing PMSE was identified for the first time. The authors suggested rapamycin as a potential therapeutic agent for seizures and cognitive impairment in PMSE patients (Bi et al. 2016). In this regard, it was previously demonstrated that five patients with PMSE treated with sirolimus (rapamycin) showed no complications, reduced seizure frequency, and improved receptive language. These findings indicate a mechanistic link between *STRADA* loss and mTORC1 hyperactivity in PMSE and suggest that mTORC1 inhibition may be a potential treatment for PMSE and other mTOR-associated neurodevelopmental disorders (Parker et al. 2013).

Similarly, in both TSC and SEGA, pharmacological treatment with mTOR inhibitors has been demonstrated to have efficacy and safety associated with tumor and seizure frequency in TSC patients, as reported in a recent meta-analysis (Li et al. 2019).

Neurofibromatosis Type 1 and Seizures

Neurofibromatosis type 1 (NF-1) is a relatively common neurocutaneous syndrome caused by a mutation in the *NF-1* tumor suppressor gene. NF-1 is a common autosomal dominant disorder affecting 1 in 2500–3000 individuals worldwide. Seizures, often focal and related to an intracranial neoplasm, occur in approximately 4–7% of patients with NF-1 (Ostendorf et al. 2013). The *NF-1* gene encodes neurofibromin 1, which acts as a GTPase-activating protein that suppresses the activity of the Ras proto-oncogene. Mutations in the *NF-1* gene are associated with high activation of the mitogen-activated protein kinase and PI3K pathways. Downstream, they are directly related to increased mTOR activity (Lau et al. 2000; Johannessen et al. 2005). Rapamycin treatment effectively reduced mTOR substrate (rpS6) phosphorylation and cell proliferation rate (Johannessen et al. 2008). However, unlike TSC, the beneficial effects of mTOR inhibitors on clinical NF-1 symptoms are unclear.

Fragile X Syndrome and mTOR Signaling

Fragile X syndrome (FXS) is one of the leading genetic causes of inherited mental retardation in males and is the second leading cause of intellectual disability after Down syndrome. Autism spectrum hyperactivity, sleep problems, anxiety, and seizures are relatively frequent in FXS (Grønskov et al. 2011).

Alterations in the *FMR1* gene that maps to the Xq27.3 band, including repetitive CGG sequence expansion (>200 triplets) in the 5'-UTR of the gene or mutations in the *FMR1* gene, are the leading cause for the disease. Loss of FMRP (fragile X protein) expression induces increased translation of several proteins, such as GluA2, PSD95, RhoA, Rac1, and matrix metalloproteinase 9, in addition to abnormalities in neuronal morphology, including substantial changes in the shape of the dendritic spine (Irwin et al. 2001). In molecular signaling, mTOR pathway activity was also upregulated in both patient-derived lymphocytes and brain tissue from FXS patients (Hoeffler et al. 2012).

Based on these mechanisms, it has been postulated that long-term treatment with mTOR inhibitors could have positive effects on clinical symptoms in FXS cases (Bhattacharya et al. 2012).

MECP2 Gene Mutations (Rett Syndrome), Seizures, and mTOR

Rett syndrome is a severe female neurological disorder caused by mutations in the *MECP2* (methyl CpG-binding protein 2) gene (Amir et al. 1999). Seizures are observed in 60–80% of affected females (Operto et al. 2019). MECP2 is an essential epigenetic factor in the brain and neurons. Loss of MECP2 activity results in abnormal biogenesis of ribosomes in the brain. Strikingly, Rett patients showed significantly increased phosphorylation of active mTORC1 or mTORC2 complexes. In the brain with MECP2 mutations, increased levels of mTOR and deregulation of its two phosphorylated forms that contribute to the activities of mTORC1 and mTORC2

are observed. The increased phosphorylation of mTORC1 in Rett patients compared with age- and sex-matched controls strongly suggest that, under normal conditions, MECP2 controls mTOR activity. These data indicate that in the human Rett brain, ribosomal RNA transcripts and mTOR-P70S6K may be elevated, pointing to a potential overactivation of this fundamental process, depleting cellular resources that are essential for other cellular functions (Olson et al. 2018)

***DEPDC5* Gene Mutations, mTOR, and Epilepsy**

DEP domain-containing protein 5 (*DEPDC5*) encodes an essential component of the GATOR1 complex, a negative regulator of the mTOR pathway. Mutations of *DEPC5* have been associated with higher rates of drug-resistant epilepsy (>50%), including forms with and without brain malformations (Klofas et al. 2020).

Using targeted next-generation sequencing in 305 patients with focal epilepsies and 91 patients with generalized epilepsies, Liu et al. recently documented that *DEPDC5* variants were related to possible mechanisms underlying phenotypical variation (Liu et al. 2020). They reported one homozygous *DEPDC5* mutation (p.Pro1031His) in a patient with focal cortical dysplasia and eight heterozygous mutations in 11 families with mild focal epilepsies, including 13 patients in eight families with focal epilepsy with febrile seizures plus/febrile seizures (FEFS+/FS).

One stop codon (p.Ser1601 Ter1604del ext133), three truncating mutations (p.Val151Serfs*27, p.Arg239*, and p.Arg838*), and four missense mutations (p.Tyr7Cys, p.Tyr836Cys, p.Pro1031His, and p.Gly1545Ser) affecting hydrogen bonds and protein stability were detected. Analysis of epilepsy-related *DEPDC5* variants revealed that MCDs exhibited a trend of higher frequency of null mutations than non-MCDs. MCD-associated heterozygous missense mutations were clustered in the structural axis for binding arrangement (SABA) domain and close to the binding sites to NPRL2/NPRL3 complex. Conversely, those associated with FEFS+/FS were far from the binding sites. Evidence from four aspects and possible evidence of subregional involvement suggested MCD and FEFS+/FS as phenotypes of *DEPDC5* variants. Moreover, these *DEPDC5* variants' phenotypes vary from mild FEFS+/FS to severe MCD. Heterozygous *DEPDC5* mutations are generally less pathogenic and commonly associated with mild phenotypes. Bi-allelic mutations and the second hit of somatic mutations, together with the genotype–phenotype correlation and subregional involvement of *DEPDC5* variants, explain severe phenotypes.

mTOR and Epileptogenesis

Regardless of mutations in genes related to the mTOR pathway, as previously described for the LADME system, overexpression of the PI3K-AKT-mTOR signaling should be considered as a consequence of seizure stress or as part of the epileptogenic process, feasible to be controlled by mTOR inhibitors.

In this regard, strong activation of mTORC1 in the brain was described in animal models of temporal lobe epilepsy (TLE), such as kainic acid (KA) or pilocarpine-induced *status epilepticus*, suggesting a central role of mTORC1 in epileptogenesis (Shima et al. 2015). The same was observed in pentylenetetrazol-induced acute seizures (Zhang and Wong 2012). Not only the increase in mTORC1-induced signaling was described in epilepsy evoked by electrical stimulation of the amygdala and angular bundle, but also inhibition of mTOR by rapamycin treatment led to a substantial reduction in seizure development despite the presence of microglia activation, suggesting that the effects of rapamycin on seizure development are not due to control of inflammation (van Vliet et al. 2012).

The potential role of mTOR in epileptogenesis was also suggested after describing a second wave of mTORC1 activation in dentate gyrus neurons but as late as 21 days after KA administration (Sha et al. 2012). A comprehensive review of the relationship between the mTOR pathway and epilepsy was described early (Cho 2011; Switon et al. 2017).

More recently, a comprehensive review of genetic variants along the PI3K-mTOR signaling pathway and GATOR1 complex (mTORopathies) associated with MCD and intractable epilepsy has been published. Furthermore, the crucial fundamental role of aberrant mTORC1 signaling in epilepsy has been indicated (Nguyen and Bordey 2021). Due to the efficacy of rapamycin treatment in seizure suppression that has been demonstrated in numerous animal models of mTORopathies, the authors suggest a targeted therapeutic strategy for epilepsy based on mTOR pathway genetic variants in each patient on a case-by-case basis. As part of the so-called precision medicine, this personalized treatment should be complemented by neuroimaging and pathological examination of brain samples to detect underlying gene variants systematically and visualize associated molecular changes.

Simultaneously, mTOR inhibition was also suggested to treat focal cortical dysplasia type II because it results from somatic brain mutations in the mechanistic target of rapamycin pathway activators mTOR, AKT3, PIK3CA, and RHEB. Also, it is a major cause of drug-resistant epilepsy. Experimentally, rapamycin reduced seizures in rodent models of DEPDC5-related epilepsy and focal cortical dysplasia type II (Moloney et al. 2021).

10.4 Conclusions

In addition to the high percentage of patients with the refractory epilepsy phenotype (30–40%), there is a high incidence of epilepsy, significantly increasing the overall number of nonresponders. Consequently, in these cases, there is a need to identify therapies guided and targeted to specific molecular markers. Thus, pharmacogenomics in epilepsy involves, at first, aspects related to PK processes (drug transporters and metabolizing enzymes) and PD processes (receptors, ion channels, enzymes, regulatory proteins, and secondary messengers) (Smolarz et al. 2021).

In the era of precision medicine, identifying these biomarkers may help better pharmacological control of any altered PK and PD processes involved in the RE phenotype.

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Chapter 11

Seizures Induce Hypoxia, and Hypoxia Induces Seizures: A Perverse Relationship That Increases the Risk of Sudden Unexpected Death in Epilepsy (SUDEP)



Jerónimo Auzmendi and Alberto Lazarowski

Abstract Hypoxia can promote both rescue-survival mechanisms and irreversible events leading to cell death. How many types of hypoxia are known, and what is the severity of the hypoxic state when more than one type of hypoxia is acting simultaneously? Assuming convulsive seizures as hypoxic-ischemic phenomena that can trigger new seizures, repetitive seizures will contribute to the development of cerebral ischemia with cell death and neurodegeneration. Generalized tonic-clonic seizures (GTCS) or *status epilepticus* will induce a systemic hypoxic repercussion affecting peripheral organs such as the cardiovascular system. This cumulative hypoxic-ischemic insult to the heart may develop into heart failure in patients with refractory epilepsy (RE). Uncontrolled GTCS increases the risk of sudden unexpected death in epilepsy (SUDEP), with a 24-fold increased risk relative to the normal population. Recently, an *epileptic heart* has been described as “a heart and coronary vasculature damaged by chronic epilepsy as a result of repeated hypoxemia” that can induce heart failure with fatal arrhythmia (bradycardia). Hypoxia induces overexpression of P-glycoprotein (P-gp), downregulation of inward rectifier potassium channels (Kir), and activation of ferroptosis, all related to membrane depolarization. These mechanisms associated with epileptogenesis and heart failure can be detected by noninvasive methods that help prevent SUDEP.

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11.1 Introduction

The first evidence of the relationship between hypoxia and seizures was described as “asphyxia with partial seizures without convulsive activity” (Jackson 1899). The first report of a case of seizure-related death that could be assumed as a sudden unexpected death in epilepsy (SUDEP) was described verbatim as follows: “He uttered a cry and was seen to be rubbing his hands together. His pulse was immediately examined for but was not palpable” (Russell 1906).

Hypoxia is a biological stimulus capable of promoting two opposite effects, rescue and survival mechanisms, or triggering a sequence of irreversible events that can lead to the death of cells, tissue, and even the patient. Brain and heart functions are critically dependent on an adequate energy supply and are highly susceptible to hypoxic conditions. A good oxygen supply is necessary for these organs to metabolize glucose as their primary energy source.

The four known types of hypoxia, and their corresponding pathophysiological consequences, are mentioned below (Fig. 11.1):

1. Hypoxic–hypoxia (Hyp–H) is caused by decreased environmental oxygen or respiratory insufficiency.
2. Anemic–hypoxia (An–H) is related to decreased functional hemoglobin.
3. Stagnant hypoxia (Stg–H) is secondary to a reduced or uneven flow of blood distribution to the tissues, resulting mainly from heart disease that impairs blood circulation.
4. Histotoxic–hypoxia (Hist–H) occurs when tissue cells undergo metabolic changes that prevent adequate use of O₂ due to poisons, cytotoxic drugs, or metabolic alterations that affect mitochondrial functioning (Pierson 2000).

Any hypoxic conditions will activate *hypoxia-inducible factor-1α* (HIF-1α), the master transcriptional regulator of cellular and developmental response to oxygen deprivation in all affected tissues. Consequently, stabilization and nuclear translocation of HIF-1α will produce upregulation of a broad spectrum of rescue genes, including the erythropoietin receptor (EPO-R), related to antiapoptotic signaling, cell proliferation/differentiation, transferrin/transferrin receptor, both related to iron transport and cell utilization. In contrast, it will induce downregulation of genes associated with opposite effects, such as hepcidin.

HIF-1α also upregulates some macrophage glycolytic enzymes to ensure a competitive bioenergetic state for M1 macrophage polarization, precursors production, and proinflammatory cytokines secretion, with a direct relationship with inflammatory responses during hypoxia (Wang et al. 2017). In this regard, astrocyte reactivation can increase the expression of ABC transporters, such as P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP-1) through TNF-α, and nuclear factor

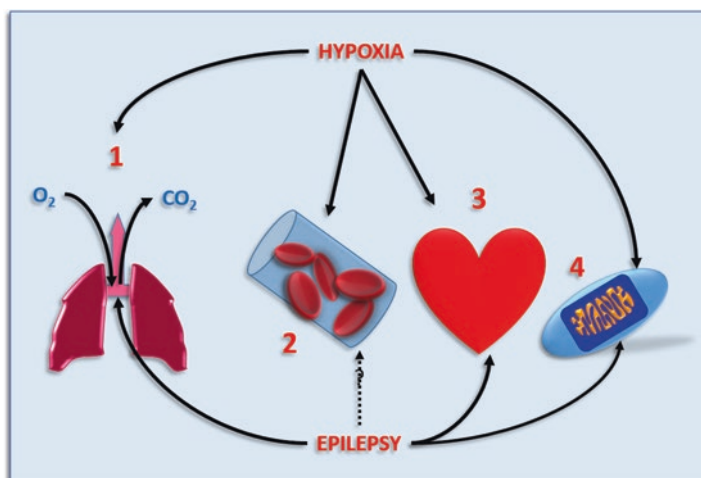


Fig. 11.1 *Ischemia*: From the Greek “ἰσχεῖν” (BRAKE) + “αἷμα” (BLOOD), ischemia is the difficulty in blood supply with increased resistance to tissue influx inducing an imbalance between oxygen supply and demand. Heart failure is also a cause of decreased coronary blood flow, unable to provide O₂ to maintain mitochondrial oxidation. During epilepsy, there may be at least three hypoxic conditions acting together. In every hypoxic condition, the result is an imbalance between oxygen demand and decreased blood flow or reduced oxygen supply/use in tissues

NF- κ B signaling, a mechanism also involved in refractory epilepsy (Wang et al. 2018) that will be discussed later in this chapter.

Interestingly, HIF-1 α also induces upregulation of several ABC transporters, such as P-gp and breast cancer resistance protein (BCRP), which are related to the multidrug resistance (MDR) phenotype and involved in the *transporters hypothesis* in RE (Tang et al. 2017; Czornyj et al. 2022; Vázquez and Fagiolino 2022).

EPO-R signaling through JAK2 activates multiple downstream pathways, including STAT5, PI3K/Akt, NF- κ B, and MAPK. These factors may play an important role in neuroprotection against brain hypoxia and neuroinflammation (Ostrowski and Heinrich 2018).

How severe are the consequences when more than one type of hypoxia acts simultaneously?

Is glutamate overstimulation a signaling pathway that activates gene expression in a HIF-1 α -dependent manner as a hypoxic condition?

Are more than one of mentioned hypoxic mechanisms active during seizures?

11.2 Hypoxia and Seizures: A Mutual Relationship of Cause and Effect

11.2.1 Hypoxia Induces Seizures and Epilepsy

The hypoxic condition is considered a necessary stress stimulus that engages the organism to develop compensatory mechanisms to ensure homeostasis. These modifications occur under hypoxia and involve several systemic responses, such as hematological, cardiovascular, renal, brain, and metabolic changes, to help the organism cope with lower O₂ levels until a normoxic condition is restored. Brain hypoxia represents the second most significant cause of sequelae of disability or death worldwide (WHO 2004).

Clinical and experimental studies have demonstrated that brain hypoxia-ischemia can induce seizures or epilepsy (Sun et al. 2001; López-Ramos et al. 2015; Wang et al. 2015; Sun et al. 2016; Shetty 2015). Also, it was suggested that hypoxia-induced seizures might play an epileptogenic role depending on their duration (Rubaj et al. 2003).

The prevalence of epilepsy increases with age, reaching up to 1% among individuals over 80 years of age (Wallace et al. 1998). During this stage of life, cerebrovascular disease (stroke) is the leading cause of epilepsy in the elderly population (Krämer 2001). Both hemorrhagic and ischemic strokes further exacerbate the development of secondary epilepsy in these elderly cases (Menon and Shorvon 2009; Gilad 2012).

After a global experimental forebrain insult, the postischemic CA3 pyramidal cells are permanently depolarized and have a reduced threshold for generating synchronized bursts. In these experiments, lower concentrations of convulsant agents, such as kainite or high K⁺, triggered all-or-none network-driven synaptic events in postischemic neurons more readily than controls, suggesting that epileptic syndromes are the neuropathological and behavioral consequences of ischemia that can be observed several months to several years after the ischemic insult (Congar et al. 2000).

Stroke (ischemic or hemorrhagic) and brain injury secondary to cardiac arrest (stagnant hypoxia) are two of the main causative factors for seizure development. However, the underlying pathophysiology of seizure development is not well understood (Mani et al. 2012).

Brain hypoxia triggers molecular processes leading to neuronal damage, where ATP production can decrease to more than 50% causing neurons to lose the ability to maintain membrane potential and undergo progressive depolarization, thus favoring a reduction of the seizure threshold (Richter et al. 2010).

Normal cerebral oxygenation may be reduced during or immediately after a seizure. Conversely, brain hypoxia may further increase seizure susceptibility. In this context, it is essential to highlight that cerebral hypoxia, either global hypoxia or focal hypoxia due to cerebrovascular accident or stroke, may have heterogeneous clinical features and arise from multiple etiologies. This complex mechanism

involved in the onset of seizures was also observed in experimental apneas (Jansen et al. 2019).

Moreover, different causes, including infectious disease as COVID-19, can lead to reduced O₂ supply. According to the Centers for Disease Control and Prevention (CDC), SARSCoV-2 is one of the viruses that could induce epilepsy or worsen the condition in epileptic patients (Kuroda 2020). Evidence suggests that seizures require an increased energy supply. Under these conditions, the brain is subjected to a relatively hypoxic environment, resulting in decreased aerobic metabolism.

Seizures arising within two weeks of the initial stroke or cardiac event are often classified as *early-onset*. Early-onset seizures are primarily observed within 24 hours of the initial insult and are considered a medical emergency, as life-threatening *status epilepticus* may develop (Kulhari et al. 2014). In addition, it has been established that around 7% of patients with stroke develop epilepsy secondary to this cerebral ischemic event (Alet et al. 2022).

Although two different types of hypoxia are involved in cardiac arrest (stagnant hypoxia) and stroke (ischemic-hypoxia), both conditions can lead to the same consequence: the development of seizures or *status epilepticus*. However, the delicate underlying mechanisms leading to convulsive episodes are not clearly understood.

Experimentally, it was demonstrated that ischemic brain hypoxia could induce early-onset seizures closely associated with severe brain injury and acute mortality in aging mice. However, prophylactic anticonvulsant treatment was able to inhibit seizure development and improve survival in these aging mice (Wang et al. 2015).

After cardiopulmonary arrest, seizures are a common problem in the intensive care unit, occurring in up to one-third of these patients during hospitalization. Furthermore, whether seizures exacerbate ischemia–hypoxia brain injury in humans remains unclear, leading to uncertainty about how aggressively they should be treated.

Similarly, the risk of developing an epilepsy scenario is increased in newborns with seizures or hypoxic encephalopathy (HIE) (Pisani et al. 2009). Especially in newborns, HIE is a frequent cause of seizures, acute mortality, or chronic neurological disability in survivors (Martinello et al. 2017; de Corrêa et al. 2022). Furthermore, it was reported that one year after a hypoxic event, cerebral palsy (31%), epilepsy (3%), or both (28%) could be observed (Allemand et al. 2009).

Moreover, during ischemic apneas (IA), respiratory impairment with oxygen desaturation has been frequently observed in patients with tonic-clonic seizures (Bruno et al. 2018; Tio et al. 2020) and was associated with a high risk of SUDEP (Vilella et al. 2019). More recently, IA and hypoxemia were detected in 10.3% of epileptic patients through respiratory polygraphy during long-term video-EEG monitoring. However, IAs are often overlooked as they are not reported by patients and are not looked for by adequate respiratory polygraphic tracking, which is highly recommended (Micalizzi et al. 2022).

Recently, it was documented that a natural scenario, such as chronic hypobaric hypoxia in high altitude areas (4000 meters), can affect the functions of the heart (Thijs et al. 2019), cerebrovascular system, and respiratory system, impair cognition, accelerate the progression of neurodegenerative diseases, and affect the normal

physiology of the organism, even leading to an increase in severe systemic diseases (West 2012).

The relationship between chronic hypoxia and epilepsy has been previously studied. It was found that chronic hypoxia can cause various pathological changes that induce increased neuronal excitability. This finding suggests that chronic hypoxia may be involved in the onset and development of epilepsy and increased susceptibility to seizures with the pharmacoresistant phenotype (Xu and Fan 2022).

What common mechanism related to O₂ deprivation is present in all these clinical syndromes, including epilepsy and refractory epilepsy? Eukaryotic cells have evolved a highly sensitive and complex mechanism capable of identifying hypoxic conditions that elicit rapid responses to rescue or induce cell death. HIF-1 α , a transcription factor initially described by Greg L. Semenza, who won the Nobel Prize 2019 for this discovery, mediates these ambivalent responses to acute and chronic hypoxia (Wang and Semenza 1993).

The maintenance of normal brain function depends on the continuous supply of oxygen. Therefore, the organism must be able to detect and respond to hypoxia rapidly. To adapt to a hypoxic microenvironment, mammalian cells activate or initiate physiological responses to hypoxia that are mediated by HIF-1 α . Moreover, long-term hypoxic responses are orchestrated by the HIF-1 α / β transcriptional complex, which plays a crucial role in cellular and systemic oxygen homeostasis.

Under hypoxia conditions, the HIF-1 α protein stabilizes and rapidly accumulates in the cytosol and then binds with the β subunit. This complex is finally translocated into the nucleus, where it serves as a transcriptional stimulation or repression of more than 100 genes (Sharp and Bernaudin 2004), which will alter the functionality of the cells that undergo modifications induced by both hypoxia and other conditions that *mimic* hypoxia, such as trauma or inflammation. Thus, after any hypoxic event, nuclear translocation of HIF-1 α is an unequivocal marker of hypoxic distress in affected cells and should be related to the increase and loss of expression of HIF-1 α up- and down-regulated genes (Wang and Semenza 1993). Erythropoietin (EPO) and erythropoietin receptor (EPO-R), vascular endothelial growth factor (VEGF), and its receptor (VEGF-R) are some of the genes upregulated by HIF-1 α under hypoxia conditions (Table 11.1) (Wang and Semenza 1996; Semenza 2000). In this regard, it was recently reported that the neocortical microvasculature of patients with drug-resistant temporal lobe epilepsy (TLE) showed increased VEGF and VEGF-R protein expression (Castañeda-Cabral et al. 2020).

Consequently, any event (injury) inducing HIF-1 α nuclear translocation should be assumed to be a condition that mimics hypoxia, even in the absence of true hypoxia. Thus, overexpression of EPO-R and VEGF-R (among others) after brain injury, even under normoxic conditions, will be markers of an underlying hypoxic-like mechanism (Table 11.1).

Traumatic brain injury (TBI) is one of the most frequent presentations in emergency departments and may be associated with seizures after the initial injury. The relationship between TBI and seizures was first described in the Edwin Smith Babylonian papyrus, dated around 1700 BC (Magiorkinis et al. 2010). However, so-called posttraumatic epilepsy (PTE) was not recognized as a clinical entity until

Table 11.1 List of genes directly upregulated by HIF1- α

<i>Glucose/Energy Metabolism and Cell Proliferation/Viability</i>
Adenylate Kinase 3
Aldolase A and C
Enolase 1 (ENO1)
Glucose Transporter 1 and 3
Glyceraldehyde-3-phosphate Dehydrogenase
Hexokinase 1 and 2
Insulin-like Growth Factor 2 (IGF-2)
IGF-Binding Protein 1 and 3 (IGFBP-1/3)
Lactate Dehydrogenase A
Phosphoglycerate Kinase 1
Pyruvate Kinase M
p21
Transforming Growth Factor β_3 (TGF β_3)
<i>Erythropoiesis and Iron Metabolism</i>
Ceruloplasmin
Erythropoietin and Erythropoietin Receptor (EPO/EPO-R)
Transferrin and Transferrin Receptor (Trf/Trf-R)
<i>Vascular Development/Remodeling and Vasomotor Tone</i>
Adrenergic Receptor (α 1B subunit)
Adrenomedullin
Endothelin-1
Heme Oxygenase 1
Nitric Oxide Synthase 2
Plasminogen Activator Inhibitor 1
Vascular Endothelial Growth Factor and its receptor (VEGF/VEGF-R)

the nineteenth century (Horsley 1886). Is TBI a hypoxic event? In this regard, using various cellular and in vivo models of TBI, it was reported that neural damage caused by TBI activates hypoxia-induced factor HIF-1 α (Bae et al. 2018; Fordington and Manford 2020).

An abstract report documented that the ABC-transporter MRP-1, but not P-gp, was highly expressed in endothelial cells, neurons, and glial cells of some TBI patients (Willyerd et al. 2012). However, the relationship with epilepsy was not mentioned. More recently, a significant increase in P-gp expression was found in a rodent model of repeated mild closed-head injuries (rmCHI) (Vita et al. 2020).

In this regard, it was documented that 4% of patients with frontal lobe epilepsy (FLE) who underwent TBI showed a worse outcome after epilepsy surgery, despite removing the epileptogenic area, and developed an RE phenotype (Gupta et al. 2014). More recently, whether RE of FLE etiology is associated with P-gp expression has been investigated. This study determined P-gp expression and cellular localization in cortical brain samples obtained from 10 patients with FLE and RE phenotypes. Five cases of PTE and five cases of FLE related to a tumor lesion (TL) were compared with autopsies of five control cases without epilepsy. P-gp studied by western blot and immunohistochemistry was overexpressed in both RE groups (PTE and TL) compared to controls (autopsies). Interestingly, P-gp expression was even higher in PTE than TL, with high overexpression in neurons (Fonseca-Barriendos et al. 2022). Not only should we consider whether cellular mechanisms induced by HIF-1 α activation in neurons can activate biological processes associated with the drug-resistant phenotype related to P-gp overexpression, but we should also determine if these mechanisms are involved in epileptogenesis.

According to this evidence, TBI should be assumed as a hypoxic event at the cellular level. If neurons are the cells involved in HIF-1 α activation, TBI could become PTE with RE phenotype. Furthermore, how long after TBI can we detect the development of secondary epilepsy? It was demonstrated that increased microglial activation could be present up to 17 years after TBI, suggesting that TBI triggers a chronic inflammatory response, particularly in subcortical regions. This highlights the importance of considering the TBI response as an evolution over time, where inflammation is a consequence of hypoxic stress (Ramlackhansingh et al. 2011).

The complex interaction between *seizures-inducing hypoxia* and *hypoxia-inducing seizures* can be evidenced by some pathological data showing that anoxic brain injury worsens with generalized tonic-clonic (GTC) *status epilepticus* (SE). A critical feature of these particular situations (hypoxia plus seizures, or seizures during hypoxia) is that the myoclonic state (MS) in a hypoxic-ischemic coma is particularly challenging, as it can be highly refractory to conventional anticonvulsants and portends an extremely poor prognosis, regardless of treatment (Hoesch et al. 2008).

11.2.2 Seizures Induce Hypoxia

Can seizures possibly activate the same HIF-1 α -dependent mechanism in the absence of O₂ deprivation?

Seizures may generate a hypoxic-ischemic state at the cerebral or even systemic level. Consistent with the idea that seizures induce a hypoxic-like condition, EPO-R was reported to be strongly immunoreactive in surgically resected mesial temporal lobe epilepsy (MTLE) hippocampi, with immunostaining localized to the luminal and abluminal plasma membrane of endothelial cells, to the endosome-like

structures of these cells, and the pericapillary astrocytic ends (Eid et al. 2004). Bratz's (1899) pioneering description of the MTLE showed that anatomical hippocampal abnormalities are characterized by neuronal loss in CA1, associated with an abundance of blood vessels in the sclerotic sector, with no pathological alterations (Bratz 1899).

Neovascularization (angiogenesis) is a compensatory response mechanism to hypoxia-ischemic stress stimulated by the VEGF and VEGF-R axis and driven by HIF-1 α (Krock et al. 2011; Dong et al. 2022). As an example of HIF-1 α -dependent neovascularization, von Hippel–Lindau (VHL) disease also has this characteristic anatomical feature as hemangioblastomas (Wizigmann-Voos et al. 1995; Semenza 2000). Under normoxic conditions, the normal VHL-protein complex binds to HIF1- α for polyubiquitination and immediate degradation by the proteasome. However, mutations in the VHL gene are an example of persistent HIF-1 α activation inducing neoplastic neovascularization.

HIF-1 α -dependent *uncontrolled angiogenesis* is a central pathological component of other chronic conditions such as many human blindness disorders, including diabetic retinopathy, age-related macular degeneration (AMD), glaucoma, and retinopathy of prematurity (ROP) (Krock et al. 2011). This evidence reinforces the concept that hypoxia or HIF-1 α -dependent mechanisms can be activated during different clinical processes, including seizure stress, or even after seizures, with functional and pharmacological consequences (Ohh et al. 2022). In this regard, a postmortem study reported that HIF-1 α is upregulated in pathological brain samples of patients with hippocampal sclerosis (Feast et al. 2012). Similar results were detected in experimental repetitive seizures and brain samples of patients with RE (Gualtieri et al. 2013).

Interestingly, downregulation miR-153 (a putative regulator of HIF-1 α) expression was identified in a microarray profile of surgically resected temporal cortex from MTLE patients. BY RT-qPCR, the downregulation of miR-153 was confirmed in plasma from MTLE patients in an independent validation cohort (Li et al. 2016). Regarding the RE phenotype, it is interesting to note that P-gp, encoded by the MDR-1/ABCB-1 gene, is also inducible by HIF-1 α (Comerford et al. 2002; Wang et al. 2019).

11.2.3 *Epilepsy Induces Hypoxia and Inflammation*

Although evidence of hypoxemia during or immediately after seizures has been described, and these seizures are associated with ictal or postictal hypoxia (Farrell et al. 2017), it has not been determined whether the observed oxygen desaturation reflects hypoventilation or is a consequence of seizure-associated peripheral vasoconstriction (Blum et al. 2000). In several RE patients with increased frequency of generalized convulsive seizures (GCS), postconvulsive central apnea (PCCA) associated with a higher risk of SUDEP may occur (Vilella et al. 2019). It was also reported that a patient with a seizure-related desaturation below 85% would have a high probability of recurrent ictal desaturations. Those RE patients with severe

seizure-related hypoxemia and hypercapnia tend to be at the highest risk for SUDEP (Bateman et al. 2008).

Whether repetitive episodes of severe seizures or *status epilepticus* could induce chronic cerebral or systemic hypoxia-ischemia has been poorly investigated (Tigaran et al. 2003; El Shorbagy et al. 2016). However, seizures were reported to generate conditions producing acute cerebral hypoxia and ischemia followed by inflammation, inducing frequent sequential events, including neurotoxicity, depolarization, inflammation, and apoptosis mechanisms, such as phosphatidylserine exposition at the outer plasma membrane (Bateman et al. 2008). Ictal hypoxemia is a standard feature, particularly in the context of generalized tonic-clonic seizures, prolonged complex partial seizures, or when antiseizure medications (ASMs) administration is reduced (Moseley et al. 2010).

A clinical report showed that the severity of postictal hypoxemia in the immediate aftermath of a generalized convulsive seizure (GCS) was more significant in temporal lobe epilepsy. However, when hypoxemia could be detected before the onset of secondary GCS, oxygen administration had a solid preventive effect (Rheims et al. 2019).

A broad spectrum of adaptive responses during hypoxia is orchestrated to help the organism cope with low oxygenation. However, hypoxia also induces the development of pathological conditions involving inflammatory processes that can lead to cell death. Under these conditions, an excessive inflammatory response affecting the brain can cause many syndromes, such as acute mountain sickness, brain inflammation and edema with microbleeds and hemosiderin accumulation, and seizures. Microglial activation is also involved in developing epilepsy with the RE phenotype (Najjar et al. 2011), subsequently inducing long-term systemic complications, including heart dysfunction leading to SUDEP.

At the cellular level, hypoxia amplifies several molecular pathways involved in phagocytosis, leukocyte recruitment, and adaptive immunity required to clear pathogens and cellular debris. Hypoxia will increase cellular oxidative stress through the production of reactive oxygen species (ROS) with deleterious effects on proteins, DNA, and lipoperoxidation leading to cell death by ferroptosis and increased production of proinflammatory cytokines, such as IL-1, IL-6, and TNF α (Fig. 11.2).

11.3 Hypoxia, Free Radicals, Iron, and Ferroptosis

11.3.1 Free Radicals and Glutathione Peroxidase System

Chronic hypoxia was recently described to increase seizure susceptibility through activation of microglia, affecting the normal functions of neurons and astrocytes by increasing the production and release of ROS, particularly superoxide anion ($O_2^{\bullet -}$) from mitochondria via transcription and phosphorylation of NADPH oxidase complex (Huang et al. 2018).

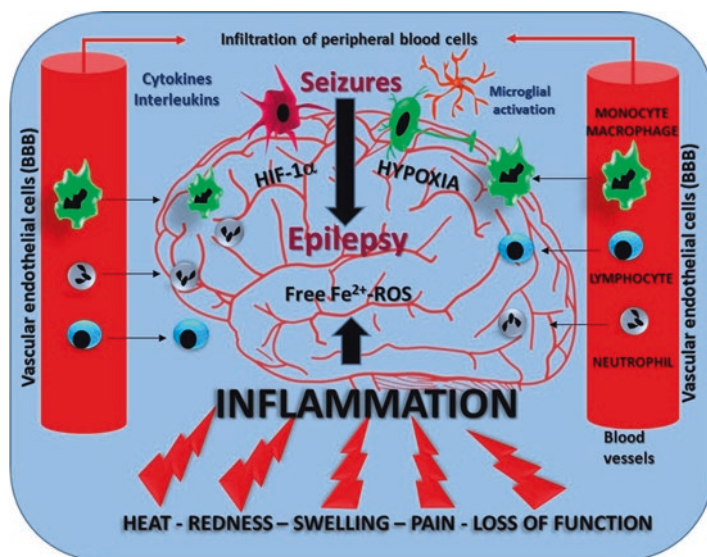


Fig. 11.2 A broad spectrum of mechanisms plays an active role in the brain during hypoxia, inflammation, and seizures. We may never know who threw the first stone

The normal NADPH oxidase enzyme complex activity depends on the continued availability of NADPH to reduce molecular oxygen to form superoxide anion, whose primary source for this enzymatic activity is the hexose monophosphate shunt. Notably, the hexose monophosphate shunt is coupled to the glutathione reductase and glutathione peroxidase (GPXs) enzymatic activity, which reduces H_2O_2 by glutathione. Additionally, upregulation of inducible nitric oxide synthase (iNOX) is observed, thus increasing the level of oxidative stress in the CNS. Furthermore, low oxygen levels lead to energy depletion and ion imbalance, followed by cell membrane depolarization, intracellular calcium (Ca^{2+}) overload, and extracellular accumulation of excitatory amino acid glutamate.

Under normal conditions, glutamate released by astrocytes synchronizes the activity of hippocampal neurons (Angulo et al. 2004). However, the expression of glutamate transporters (EAAT1 and EAAT2) in astrocytes—an essential mechanism to regulate extracellular glutamate concentration and prevent glutamate excitotoxicity—is rapidly reduced following hypoxia and ischemia, leading to increased accumulation of extrasynaptic glutamate (Ketheeswaranathan et al. 2011).

Overstimulation of glutamate receptors due to elevated glutamate concentration in the extracellular space leads to a massive Ca^{2+} influx. Consequently, it triggers a broad spectrum of signaling pathways, including oxidative stress, free radicals, lipid and protein oxidation, DNA damage, mitochondrial dysfunction, and protease activation, overall resulting in cell membrane depolarization (lowering the seizure threshold), and release of more neurotransmitters from presynaptic terminals (Belov Kirdajova et al. 2020).

Microglial activation (MAP) was detected with routine staining (hematoxylin and eosin) in 50% (46) of brain samples from 319 surgically treated epilepsy cases, with mild (69.6%), moderate (26.1%), and severe (4.3%) activations. The prevalence and severity of MAP were independent of underlying abnormalities. Immunomodulatory therapy was followed by a reduced seizure activity of greater than 90% in treated patients, suggesting that microglial activation and proliferation initiate a cycle of *inflammation-induced seizures* and *seizure-induced inflammation* (Fig. 11.2) (Najjar et al. 2011).

Murphy et al. reported that glutamate triggered Ca^{2+} -dependent cell death by inhibiting cystine import via the cystine/glutamate antiporter system Xc^- in a neuroblastoma cell line, where a significant depletion of the critical antioxidant glutathione (GSH) and increased oxidative stress was observed (Murphy et al. 1989). Accordingly, it was shown that an active system Xc^- could modulate neuroinflammation, as the absence of a functional system Xc^- favors the anti-inflammatory (M2) over the proinflammatory (M1) microglial phenotype (Mesci et al. 2015). Transcription of xCT, the specific subunit of the system Xc^- , is enhanced by the presence of ROS and inflammatory cytokines, indicating that the system Xc^- could be involved in the excitotoxic extracellular glutamate release in neurological disorders associated with increased oxidative stress and neuroinflammation (Massie et al. 2015), suggesting having direct consequences on glutathione synthesis and epileptogenesis. The system Xc^- could be an exciting target for pathologies associated with excessive extracellular glutamate release in the hippocampus, such as epilepsy. Consistent with these findings, xCT deletion in mice was reported to elevate the threshold for limbic seizures and abolish the proconvulsant effects of N-acetylcysteine (De Bundel et al. 2011).

The potential involvement of neuroinflammation and oxidative stress with epileptogenesis and RE phenotype has been recently reviewed (Geronzi et al. 2018; Vezzani et al. 2019). Posttranslational oxidative modifications can directly influence the function of crucial neuroinflammatory mediators through the transcriptional regulators NF κ B and Nrf2 (activated by ROS) (Fabisiak and Patel 2022). This interaction between ROS and neuroinflammation has been recently described also in posttraumatic epileptogenesis (Eastman et al. 2020).

One of the cellular mechanisms of protection against oxidative stress lies in the action of the antiporter system Xc^- that mediates the exchange of extracellular cystine with intracellular glutamate. When cystine is internalized, it is reduced to cysteine, one of the precursors of GSH biosynthesis (Rochette et al. 2022). These observations suggest that overstimulation by glutamate due to hypoxic conditions, convulsive stress, or both can inhibit GPX4-dependent antioxidant action, leading to ferroptosis—a form of cell death implicated in a wide variety of human diseases. This mechanism can be detected by elevated iron accumulation in cells as hemosiderin deposits (Manabe et al. 2020).

Ferroptosis is characterized by excessive phospholipid (PL) peroxidation of membranes rich in polyunsaturated fatty acids (PUFAs) via an iron-dependent mechanism leading to cell death and the accumulation of hemosiderin (Hassannia

et al. 2019). This lipid peroxidation mechanism highlights the essential roles of free iron as Fe^{2+} and ROS initiated by nonenzymatic Fenton reactions, lipid peroxidation, and enzymatic mechanisms such as lipoxygenases (LOXs) (Tang et al. 2019). However, enzymatic action is also observed in arachidonic acid (AA)—a significant component of cell membrane lipids—which can be metabolized into prostaglandins (PGs) via the cyclooxygenase (COX) pathway. Recent research shows that ferroptosis can directly increase the expression of the prostaglandin-endoperoxide synthase 2 (*PTGS2*) gene, which encodes COX_2 , accelerates the metabolism of AA, and promotes the secretion of inflammatory signaling molecules (Yang et al. 2014).

Ferroptosis has also been described recently in epileptic patients (Cai and Yang 2021; Petrillo et al. 2021; Zimmer et al. 2021), experimental epileptic models (Mao et al. 2019; Jia et al. 2020), and experimental *status epilepticus* (SE) (Du et al. 2022). Our research group described ferroptosis in cardiomyocytes after pilocarpine-induced SE associated with heart dysfunction and SUDEP (Akyuz et al. 2021). Glutamate-cysteine ligase (GCL), also known as gamma-glutamylcysteine synthetase, is the first rate-limiting enzyme of glutathione synthesis (Fig. 11.3):

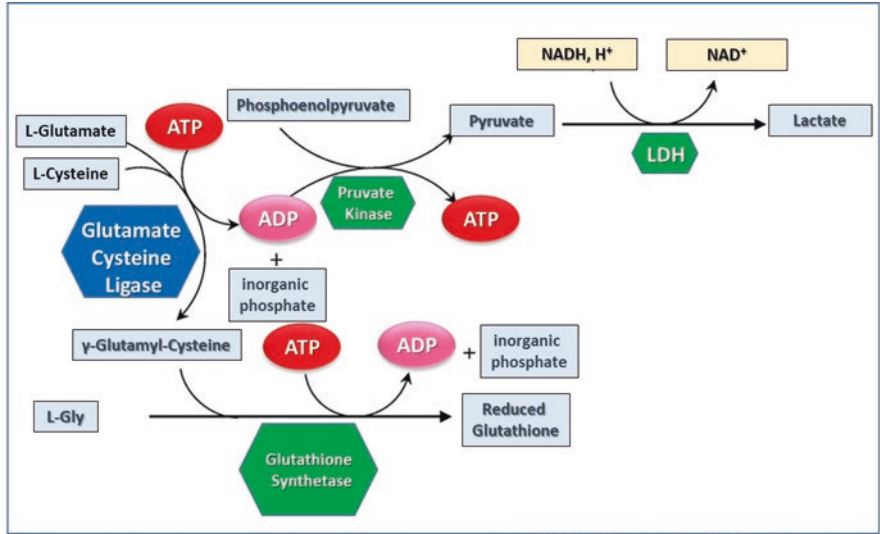
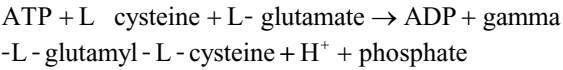


Fig. 11.3 Enzymatic pathways showing the central role of glutamate-cysteine ligase (GCL) in glutathione synthesis

11.3.2 Hypoxia, Free Radicals, and Induction of ABC Transporters

The GCL enzyme comprises a catalytic subunit (GCLC, ~73Kd) and a modifying subunit (GCLM, ~30Kd). Antioxidant-response element (ARE) and activator protein 1 (AP-1) are two cis-acting elements present in the promoter of both human glutamate-cysteine ligase (GCL-C and M) subunits genes. Nrf2 binds and trans-activates the ARE present in the human GCLC and GCLM promoters. Mice lacking Nrf2 also exhibit lower GSH levels (Yang et al. 2005).

The glutathione system is regulated by the inducible nuclear factor erythroid-2-related factor 2 (Nrf2), which is repressed by epigenetic inactivation of its promoter during neuronal development. Nrf2 neutralizes ROS to maintain cellular redox balance (Fig. 11.4). Considering that neurons lose proliferative properties and must survive for many years without becoming dysfunctional due to the accumulation of oxidative damage, it is paradoxical that these cells are less endowed with antioxidant defenses compared to astrocytes (Bell et al. 2015).

Mechanisms, such as oxidative stress, neuroinflammation, and hypoxia, are involved in the development of epileptogenesis and the RE phenotype due to the overexpression of ABC transporters (Singh et al. 2010). These mechanisms are directly related to a neuroinflammatory condition associated with an increased

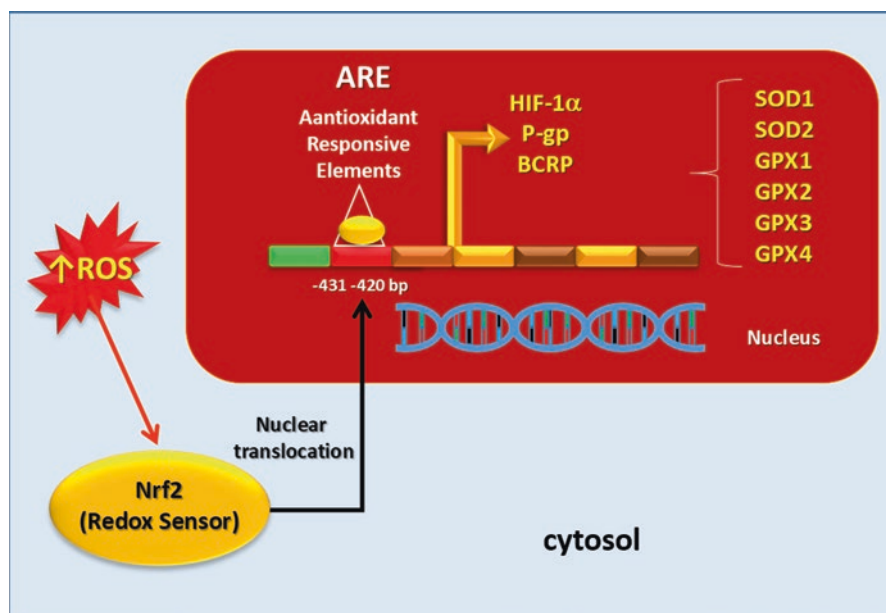


Fig. 11.4 Nrf2 is a ROS-activated transcription factor that translocates to the nucleus and also promotes the upregulation of antioxidant genes, such as SOD and GPX isoforms, HIF-1 α , and ABC transporters, such as P-gp and BCRP

COX₂ expression (Kobylarek et al. 2019), which was shown to upregulate P-gp expression in BBB cells under convulsive stress (Bauer et al. 2008). The same P-gp overexpression was also observed in neurons under convulsive or hypoxic conditions (Ramos et al. 2004; Lazarowski et al. 2006; Merelli et al. 2018).

Glutathione peroxidase-4 (GPX4) uses GSH as a cofactor to reduce *toxic lipid peroxides* into their respective alcohols. Therefore, depletion of either GSH or GPX4 will increase lipid peroxides that will damage the cell membrane and lead to *iron-dependent cell death* or ferroptosis (Fig. 11.5).

Glutamate is the most abundant free amino acid in the CNS; it acts as an excitatory neurotransmitter and is released by astrocytes to synchronize the activity of hippocampal neurons. Glutamate accumulated in the extracellular space (ECS) activates glutamate receptors on the surface of postsynaptic terminals (Angulo et al. 2004; Rowley et al. 2012).

Several reports have shown that HIF-1 α is upregulated in the pathological hippocampus of patients with temporal lobe epilepsy (Feast et al. 2012; Gualtieri et al. 2013). However, the mechanism inducing this upregulation of HIF-1 α has not been elucidated.

Although hypoxia induces glutamatergic excitotoxicity, the counter mechanism has not been thoroughly investigated. Excess glutamate is suspected of generating a neuronal response that activates signaling pathways similar to ischemic conditions with stabilization and activation of HIF-1 α despite not being in actual hypoxia

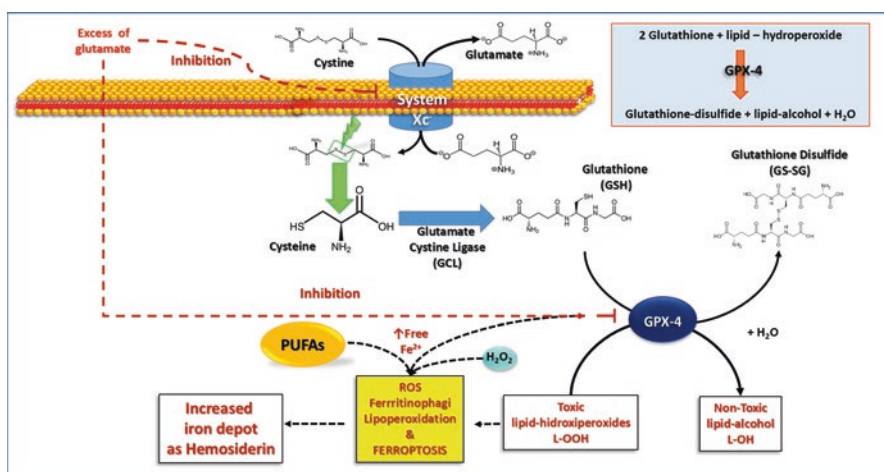


Fig. 11.5 Cystine/glutamate antiporter system Xc⁻. Phospholipid peroxidation mediated by the cyst(e)ine/GSH/GPX4 regulatory pathway induces ferroptosis death with hemosiderin accumulation

An excess of this excitatory amino in the intrasynaptic space will bind to glutamate receptors, the opening of which leads to additional Ca^{2+} entry into neurons. This sustained excess activation of Ca^{2+} -permeable channels due to impaired glutamate uptake by astrocytes can occur secondary to hypoxia but also during seizures (without hypoxia). In both conditions, excitotoxicity with neuronal oxidative stress will develop.

Oxidative stress with high ROS production is essential in several brain pathologies, including epilepsy. Excessive glutamate release with subsequent glutamatergic neuronal stimulation leads to increased ROS production generated by NADPH oxidase (Hernández-Espinosa et al. 2019), inducing oxidative stress, excitotoxicity, and neuronal damage, as previously demonstrated in cultured neurons (Reynolds and Hastings 1995; Savolainen et al. 1998).

Moreover, it was demonstrated that under normoxic conditions during acute glial inflammation induced by *in vitro* administration of $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and $\text{IFN-}\gamma$, HIF-1 α mRNA expression levels were significantly upregulated (de Lemos et al. 2013). These experimental data strongly suggest that HIF-1 α can be activated secondary to inflammatory stress, without O_2 deprivation, inducing the progressive development of a hypoxia-like condition that accompanies the duration of the inflammatory process and upregulates the genes previously described. To confirm this observation, our group recently demonstrated that excitotoxic stress (300 μM glutamate, 5 min) induced overexpression of EPO-R and P-gp simultaneously with nuclear translocation of HIF-1 α and NF κB in primary cortical neurons cultured under normoxic conditions (Merelli et al. 2019). These results demonstrated that glutamate overstimulation mimics intracellular hypoxic conditions without actual hypoxia.

Furthermore, this report also documented that primary astrocytes exposed to chemical hypoxia with CoCl_2 (0.3 mM, 6 h), which induces P-gp overexpression as well (Caltana et al. 2009), showed increased efflux of the P-gp-specific fluorescent substrate rhodamine-123 (Rho-123). Administration of recombinant human erythropoietin (rHu-EPO) inhibited Rho-123 efflux similar to that observed by the P-gp-specific inhibitor tariquidar, demonstrating for the first time that rHu-EPO can inhibit P-gp activity. These results suggest that EPO may be a new therapeutic tool in RE and also protect against the ischemic-neurodegenerative process (Merelli et al. 2015; Merelli et al. 2019), as our group previously demonstrated by exogenous nasal administration of pharmacological doses of rHu-EPO in experimental focal brain hypoxia (Merelli et al. 2011).

Since O_2 acts as a final electron acceptor in the mitochondrial electron transport chain to generate ATP in eukaryotic cells, a close relationship with tissue inflammation will develop during O_2 deprivation (hypoxia). This evidence indicates that hypoxia and inflammation are two intimately related and potentiated mechanisms that may be inducing P-gp overexpression through HIF-1 α and COX_2 (Fig. 11.6).

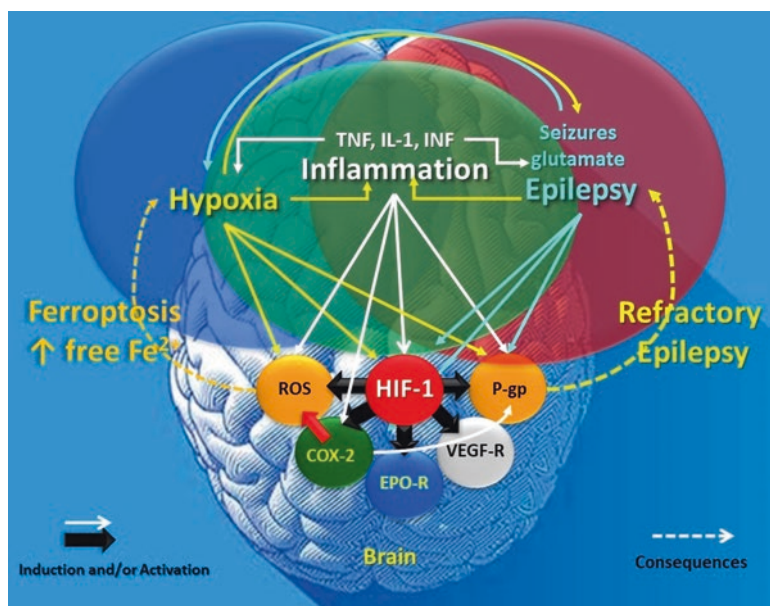


Fig. 11.6 Overlapping cellular mechanisms activated by different stress insults. Hypoxia, Inflammation, and Seizures. Are they different?

11.4 Refractory Epilepsy, Systemic Hypoxia, Epileptic Heart, and Sudden Unexpected Death in Epilepsy

11.4.1 Cardiac Effects of Refractory Epilepsy

Patients with epilepsy often experience repetitive acute seizures or seizure clusters, negatively impacting the quality of life, emotional well-being, daily function, and productivity of patients and their caregivers. These seizure clusters are characterized by more than two seizures occurring between 6 to 24 hours (Jafarpour et al. 2019).

After the pioneer and potential first description of sudden death during an epileptic crisis (Russell 1906), sudden unexpected death in epilepsy (SUDEP) should be assumed as a very likely “final chapter of a convulsive life” for a group of patients with RE with no seizure control under therapy with several recommended antiseizure medications (ASMs). Some risk factors for SUDEP have been identified, such as repetitive generalized tonic-clonic seizures (GTCS), male sex, poor compliance with ASMs prescription, young age, early age at seizure onset, or being bedridden at the time of death (Devinsky 2011). In these patients, a multidrug-resistant phenotype usually develops in which brain P-gp overexpression is one of the most frequent findings (Lazarowski et al. 2007a).

SUDEP determination excludes sudden cardiac death as, by definition, it does not include known causes of mortality, such as cardiac comorbidities (Walczak et al. 2001). In addition to the SUDEP definition, an *epileptic heart* has been recently defined as “a heart and coronary vasculature damaged by chronic epilepsy as a result of repeated hypoxemia with increased catecholamines leading to electrical and mechanical heart dysfunction” (Verrier et al. 2020). This concept was supported by clinical evidence showing a higher percentage of heart disease in patients with chronic epilepsies compared to patients with no epileptic history (Zack and Luncheon 2018). Chronic therapy with several ASMs must be considered unsuccessful in patients with chronic epilepsies. Perhaps the most severe epilepsy-related stress could lead to the development of Takotsubo syndrome (TKS), whose possible relationship to SUDEP has been suggested (Dupuis et al. 2012).

In two experimental studies of chronic and acute cardiac hypoxia-ischemia, a significant loss of cardiac retention of ^{99m}Tc -2-methoxyisobutylisonitrile (^{99m}Tc -SESTAMIBI) in the affected ischemic regions of the heart and a concomitant high expression of P-gp associated with cardiac stunning have been demonstrated (Lazarowski et al. 2005; Laguens et al. 2007). Cardiac stunning was also documented and included in this spectrum of stress-related cardiomyopathies in TKS (Ancona et al. 2016). Moreover, in this condition, cardiac hypoxic-ischemia was postulated (Ghadri et al. 2015).

Both acute and chronic seizures can cause cardiac ischemia and develop several effects on the heart, such as altered heart rate variability, ST-segment depression, cardiac fibrosis, increased heart rate, or severe bradycardia. Transient ischemia in the heart may be observed after each seizure. However, if seizures are frequent, this temporary condition could develop into chronic heart ischemia (Tigaran et al. 2003; Nei et al. 2012).

Intermittent hypoxia is broadly defined as repeated episodes of hypoxia interspersed with episodes of normoxia. The actual experimental protocols vary in cycle length, number of hypoxic episodes per day, and number of days of exposure (Neubauer 2001). Chronic intermittent hypoxia (CIH) is the most distinct feature of obstructive sleep apnea (OSA), a common breathing and sleep disorder that leads to several neuropathological consequences, including alterations in the hippocampal network and high susceptibility to seizures. In this respect, 21 days of CIH increased gamma-band hippocampal network activity and aggravated 4-aminopyridine-induced epileptiform activity in adult rats, which remitted after 30 days of normal oxygenation (Villasana-Salazar et al. 2020).

In an experimental model of intermittent hypoxia (IH) during sleep, brain upregulation of both HIF-1 α and P-gp was demonstrated, which was also detected in the heart of these rats (Aviles-Reyes et al. 2010). Previously, after injection of CoCl_2 —a chemical compound to induce hypoxia—into the cerebral cortex of rats, nuclear translocation of HIF-1 α was detected concomitant with P-gp expression in both neurons and vascular endothelial cells, together with the expression of the erythropoietin receptor (EPO-R), a classic HIF-1 α responsive gene (Lazarowski et al. 2007b; Caltana et al. 2009; Merelli et al. 2011). Increased P-gp expression was also observed in the brain and heart after repetitive PTZ-induced seizures, followed by a

high ratio of spontaneous death, suggesting a potential relationship with SUDEP (Auzmendi et al. 2014).

Furthermore, repeated experimental episodes of pilocarpine-induced *status epilepticus* (Pilo-SE)—one per week—resulted in nuclear translocation of HIF-1 α and high expression of P-gp in cardiomyocytes. These findings were associated with electrocardiographic (ECG) changes, such as prolonged QT intervals, bradycardia, and a high rate of spontaneous death observed at least 3 days after each Pilo-SE (Auzmendi et al., 2018).

Another preliminary experimental study developed under the same conditions in rats found a significant reduction in ^{99m}Tc -SESTAMIBI heart retention 72 h after Pilo-SE consistent with elevated P-gp expression in cardiomyocytes and associated with a high rate of sudden death (Fig. 11.7) (Auzmendi et al. 2017).

Although the mechanism that triggers sudden death is unknown, evidence suggests that SUDEP may originate from heart failure due to elevated hypoxic stress

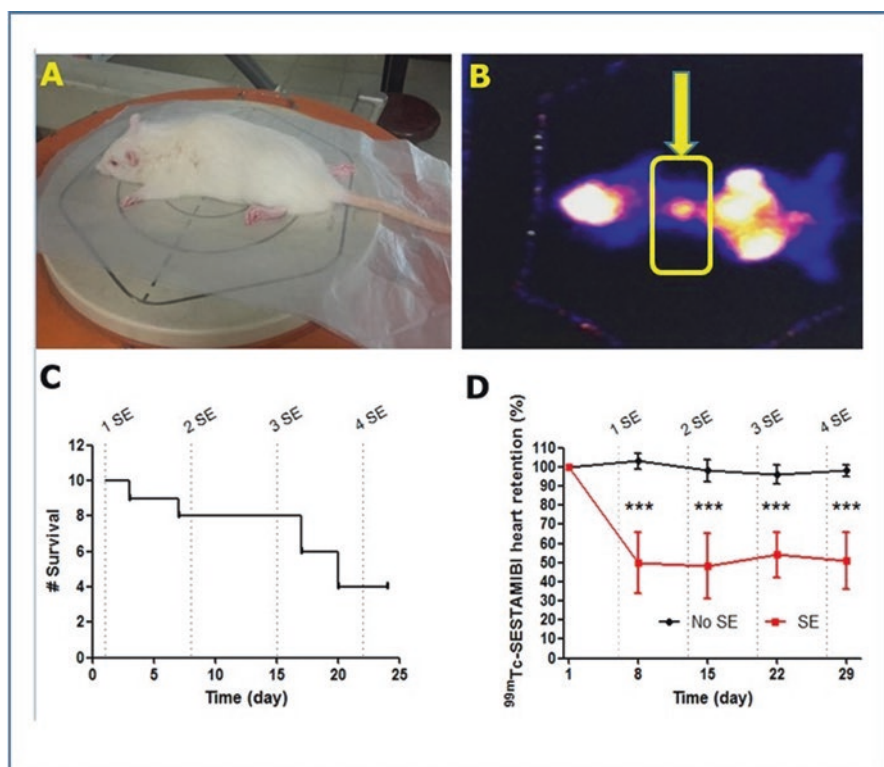


Fig. 11.7 (a) Image of rat on the SPECT gamma camera. (b) Arrow indicates ^{99m}Tc -SESTAMIBI heart retention. (c) Survival of rats after pilocarpine-induced SE. (d) Reduced ^{99m}Tc -SESTAMIBI heart retention 72 hours after pilocarpine-induced SE in rats (red line) associated with increased spontaneous death (c), as compared with controls (black line) (***) ($p < 0.001$) (Auzmendi et al. 2017)

and sympathetic overstimulation that results in neurocardiogenic injury. This affects the myocardium's electrical properties, leading to heart failure with fatal arrhythmia (bradycardia). On this basis, it is suggested that the highly cumulative burden of convulsive stress results in a hypoxic cardiac insult, where P-gp expression may play a depolarizing role in cardiomyocyte membranes.

Other molecular regulators of membrane potential in neurons and cardiomyocytes are the inwardly rectifying potassium (Kir) channels, genetic variants related to epilepsy, and heart dysfunctions (Feng et al. 2017; Staruschenko et al. 2022). Kir channels control cellular excitability in the heart by acting toward the cardiac action potential repolarization phase (Anumonwo and Lopatin 2010). Based on this property, it would be logical to assume that Kir channel dysfunction or absence is a deleterious mechanism conferring increased durability to cardiomyocyte membrane depolarization induced by hypoxia and favored by P-gp overexpression, as mentioned above. Indeed, after PTZ-induced repetitive seizures in rats, molecular analyses showed a significant decrease in cardiac Kir channel mRNA and protein expression (Akyüz et al. 2018), associated with an overexpression of HIF-1 α in these cardiomyocytes, thus suggesting an association with SEDEP (Auzmendi et al. 2021).

Since the erythropoietin receptor (*EPO-R*) gene is also a hypoxia-inducible gene, it has been suggested that the exogenous administration of erythropoietin could have an antiapoptotic and rescue effect not only in the brain but also at the cardiac level (Merelli et al. 2019; Auzmendi et al. 2020). Importantly, P-gp is typically absent in neurons and cardiomyocytes, and hypoxia-induced P-gp expressions in these cells are not only related to its pharmacoresistant property. Pioneering studies have shown that P-gp can modify the resting membrane potential, producing depolarization with values of -70 to -10 mV in expressing cells (Wadkins and Roepe 1997; Merelli et al. 2019).

These specific observations are consistent with the properties of ^{99m}Tc -SESTAMIBI, that although it binds to the mitochondrial membrane, it is released outside the cell by P-gp under membrane depolarization (Piwnicka-Worms et al. 1990). This depolarization has also been observed in the hippocampus and neocortex after repetitive PTZ-induced seizures in rats where P-gp was highly expressed (Auzmendi et al. 2013). These data suggest that seizures induce a hypoxic condition in the brain and the heart, with increased P-gp expression and simultaneous loss of Kir expression in cardiomyocytes. These up-and-down regulations play a central role in developing sustained depolarization of cardiomyocytes, leading to potentially fatal heart failure after a new seizure. This situation can be detected by myocardial perfusion single-photon emission computed tomography (SPECT) imaging using ^{99m}Tc -SESTAMIBI, a tracer of functional P-gp expression in cardiomyocytes (Auzmendi et al. 2018). Reduced ^{99m}Tc -SESTAMIBI heart retention is a hallmark of cardiac depolarization and ischemia and could be a noninvasive predictor of high risk for SUDEP in patients with RE.

11.4.2 Heart Ferroptosis and SUDEP

Terminal cardiac arrhythmia in SUDEP may develop as a result of a high rate of seizure-induced hypoxic stress with sympathetic overstimulation triggering a neurocardiogenic injury, recently termed *epileptic heart*: a heart and coronary vasculature damaged by chronic epilepsy as a result of repeated catecholamine surges, and hypoxemia leading to electrical and mechanical dysfunction. The *epileptic heart* is characterized by heart ischemia, heart rhythm disturbances, cardiac electrical instability including T-wave alternans (TWA)—periodic beat-to-beat variation in the amplitude or shape of the T wave in an ECG—bradycardia, prolonged QT intervals, and cardiac stunning (Verrier et al. 2020, 2021).

Experimental data from a Dravet syndrome model showed that cardiomyocytes exhibited increased excitability, prolonged action potential duration, and triggered activity. In this model, continuous radiotelemetric electrocardiographic recordings showed QT-interval prolongation, ventricular ectopic foci, idioventricular rhythms, beat-to-beat variability, ventricular fibrillation, and focal bradycardia that were associated with spontaneous deaths in mice (Auerbach et al. 2013).

Concerning oxidative stress, it was recently shown that ubiquitin-specific peptidase 15 (USP15) expression was upregulated in a PTZ-kindled rat model. USP15 upregulation was associated with increased intracellular ROS levels and enhanced superoxide dismutase (SOD) activity (Chen et al. 2020). SOD, which converts superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and dioxygen (O_2), may act through a reaction termed disproportionation as the front line of defense against ROS-mediated injury (Kangralkar et al. 2010).

Due to repetitive oxidative stress in the heart, iron overload cardiomyopathy (IOC) results from iron accumulation in the myocardium, elevated ROS production, lipid peroxidation, and hemosiderin accumulation as the final biomarker related to cardiomyocyte ferroptosis with hemosiderin accumulation. Furthermore, iron overload cardiomyopathy is the leading cause of death in patients with iron overload secondary to hereditary hemochromatosis or chronic blood transfusion therapy in thalassemia major (Kremastinos and Farmakis 2011).

Irrespective of the above description of brain ferroptosis in epilepsy, our group first described iron accumulation in the heart using the experimental models PTZ-kindling and pilocarpine-induced status epilepticus. Following SUDEP-related repetitive GTCS, we observed increased cardiac HIF-1 α expression, indicating hypoxic-ischemic damage, EPO-R overexpression, and hemosiderin accumulation, which may act as an underlying mechanism contributing to the development of end-stage cardiac arrhythmia in SUDEP. Since noninvasive imaging methods can detect tissue iron accumulation, cardiac iron overload in patients with RE could be treated with chelation therapy to reduce the risk of SUDEP (Akyuz et al. 2021).

Iron can be stored in myocytes as ferritin, hemosiderin, and labile cell iron (free iron) in IOC. The latter is the most active, but hemosiderin can be detected by imaging studies such as cardiovascular magnetic resonance T2* (CMR-derived T2*). This noninvasive imaging method has revolutionized the clinical management of

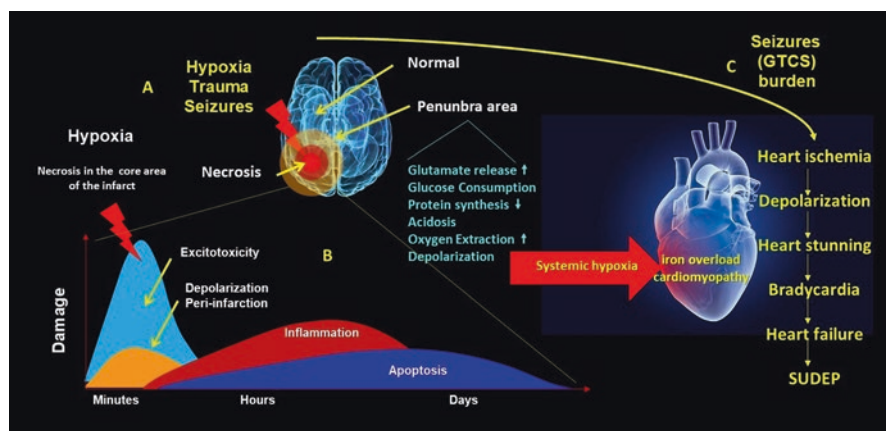


Fig. 11.8 Seizures assumed as hypoxic/ischemic insults (a) inducing a sequence of events at the brain level. (b) beginning with excitotoxicity, depolarization, inflammation, and neuronal apoptosis. (c) Systemically, a repetitively high load of convulsive stress (GTCS) will affect the myocardium with ischemia, depolarization, stunning, bradycardia, functional failure, and finally, SUDEP

patients with hemoglobinopathies and other iron overload conditions (He 2014). CMR-derived T2* allows accurate diagnosis and quantification of myocardial and hepatic iron deposition, thus tailoring and monitoring iron chelation therapy, improving survival, such as in thalassemia major cases (Modell et al. 2008). Overall, EEG monitoring with LQT detection, bradycardia, and cardiac imaging tools (^{99m}Tc -SESTAMIBI-SPECT and CMR-derived T2*) are the “tell-tale heart” (Edgar Allan Poe) tools that alert us of the high risk of SUDEP in patients with refractory epilepsies (Fig. 11.8).

11.5 Conclusions

As described, hypoxia, seizures, and inflammation trigger cellular response mechanisms common to each other. It is not always clear who gave rise to whom, hypoxia to seizures or seizures to hypoxia, and the same interaction occurs with neuroinflammation. In all cases, the result is HIF-1 α nuclear translocation, inducing overexpression of ABC transporters, mainly P-gp, responsible for the drug-resistant phenotype, and EPO-R as a marker of that hypoxic condition.

Several lines of evidence have linked EPO to an antiapoptotic role in both CNS and heart tissue due to an antiapoptotic role of the EPO/EPO-R signaling axis under hypoxia conditions, such as refractory epilepsy and heart failure. Because of its elevated expression in the brain and heart after hypoxia and seizures, EPO-R appears to contribute to tissue protection or regeneration under EPO stimulation. Moreover, these protective effects could represent a new field of research and a novel

therapeutic strategy for the early treatment of these conditions and the prevention of SUDEP (Auzmendi et al. 2020).

Severe seizures will impact the peripheral level and decrease heart oxygen homeostasis, inducing P-gp overexpression and Kir channel downregulation, which affects therapeutics and leads to membrane depolarization. Simultaneously, these conditions will cause iron-dependent cell death (ferroptosis) with hemosiderin accumulation, avoidable by iron chelation, and finally, EPO-R upregulation that will allow a novel therapeutic strategy with EPO.

Together, these strategies could counteract the oxidative stress generated by hypoxia/seizures with cerebral and cardiac protection and decreased risk of SUDEP.

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Chapter 12

Neonatal Excitotoxicity Triggers Degenerative Processes Related to Seizure Susceptibility and Pharmacoresistance



Mónica E. Ureña-Guerrero, Antoni Camins-Espuny, and Carlos Beas-Zárate

Abstract Neuronal damage and seizures are two closely related events, not only reciprocally as cause and effect but also through the cellular mechanisms and signaling pathways that they share throughout the degenerative processes that trigger them or are triggered by them. Therefore, increases in extracellular levels of the excitatory neurotransmitter glutamate, overactivation of its receptors, excessive neuronal excitation, and neuronal death by excitotoxicity have been described as part of these processes. Our group has shown that the excitotoxicity induced by monosodium glutamate (MSG) in the early stages of development produces significant modifications in the glutamatergic and GABAergic neurotransmission systems. In addition, an increased seizure susceptibility in adulthood has been observed after neonatal MSG treatment, particularly when the potassium channel blocker 4-aminopyridine or the gamma-aminobutyric acid (GABA) antagonist iodide-methyl-bicuculline is used as convulsive drugs, but not when the selective glutamate agonist N-methyl-D-aspartate (NMDA) is used. Throughout this chapter, the topics mentioned above and the hypothesis that neonatal excitotoxic damage can induce some type of drug resistance to NMDA analogs will be discussed in detail.

Keywords Excitotoxicity · Monosodium glutamate · Seizure susceptibility · NMDA receptor · Pharmacoresistance

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12.1 Introduction: The Relationship Between Excitotoxicity and Seizure Susceptibility Through Amino Acid Neurotransmitters

Although more than a hundred substances appear to act as neurotransmitters, two small molecules are particularly important in the central nervous system (CNS) of mammals: glutamate and GABA (gamma-aminobutyric acid); both are highly concentrated amino acids in the brain and are also biochemically related to each other, but in general, in adulthood, they have opposite effects on neuronal activity (Purves et al. 2001; Hassel and Dingledine 2006; Olsen and Betz 2006; Deutch and Roth 2008). Glutamate is a dicarboxylic amino acid negatively charged at physiologic pH, synthesized by the phosphate-activated glutaminase (PAG) enzyme that hydrolyzes the amine group of the glutamine in a phosphate-dependent manner, and it is considered the main excitatory neurotransmitter in the nervous system of vertebrates (Hassel and Dingledine 2006; Rowley et al. 2012). Also, recently glutamate has been proposed as a metabolic hub linking glucose and amino acid metabolism with synaptic transmission (Andersen et al. 2021). In contrast, GABA is a neutral amino acid, synthesized by the glutamic acid decarboxylase (GAD) enzyme that hydrolyzes the alpha-carboxyl group of glutamate, and it is considered the main inhibitory neurotransmitter in the nervous system of mature mammals (Olsen and Betz 2006; Rowley et al. 2012). Both glutamate and GABA are considered as classical neurotransmitters because the mechanisms involved in its synthesis, vesicular packing, release, postsynaptic receptors, synaptic inactivation, and neuronal pathways have been clearly identified in the CNS (Deutch and Roth 2008; Rowley et al. 2012). In addition, receptor specifically sensitive to each of the two neurotransmitters coexist in virtually all structures, regions, and developmental stages of the CNS (Ben-Ari 2001; Manet and Represa 2007; Deutch and Roth 2008; Aronica et al. 2011) and, interestingly, it has been shown that also GABA and glutamate can be co-released in some synapses (Gundersen 2008; Root et al. 2014). However, a particular consideration must be made, in early stages of development, when neurons have not yet established definitive synaptic contacts, GABA induces neuronal excitation and exerts trophic effects by mechanisms that include both the reverse electrochemical potential of chloride and extrasynaptic GABA_A receptors (Ben-Ari 2001; Ben-Ari et al. 2007; Jensen 2011; Cellot and Cherubini 2013).

According to the essential roles of GABA and glutamate, it is evident that any significant alteration in the dynamic balance between them could lead to some pathological conditions (Martisova et al. 2012; Rowley et al. 2012; Andersen et al. 2021; Sarlo and Holton 2021; Sood et al. 2021). Thus, both experimental and clinical trials have confirmed the hypothesis that an increase in glutamate-mediated neuronal excitation or a deficiency in GABA-mediated neuronal inhibition in adulthood could increase the risk of seizures, and it is related to epilepsy (Mares and Kubová 2008; Werner and Coveñas 2011; Rowley et al. 2012; Sood et al. 2021). In general, seizures have been associated with elevated glutamate levels or reduced GABA levels in the brain (Morales-Villagran and Tapia 1996; Tapia et al. 1999;

Wilson et al. 1996; López-Pérez et al. 2010; Sarlo and Holton 2021). Also, seizures can be induced by glutamate agonists (Kohl and Dannhardt 2001; Vincent and Mulle 2009) and controlled by their antagonists (Morales-Villagran et al. 1996; Kohl and Dannhardt 2001). Otherwise, seizures can be promoted or diminished by GABA antagonists (Sperk et al. 2004; Löscher 2011) or agonists (Tolman and Faulkner 2009; Biagini et al. 2010), respectively. In addition, several alterations in the glutamatergic and GABAergic neurotransmissions also seem to be linked to the seizure activity (Treiman 2001; Mares and Kubová 2008; Werner and Coveñas 2011; Rowley et al. 2012; Sood et al. 2021). At this point, it is important to clarify that although GABA and glutamate play a fundamental role in seizure activity, other neurotransmitters and neuromodulators also have relevant implications in epilepsy (Manent and Represa 2007; Mares and Kubová 2008; Biagini et al. 2010; Werner and Coveñas 2011), one of the most complex neurological disorders. Furthermore, because GABA-mediated neuronal excitation seems to be a triggering condition for neonatal seizures (Ben-Ari et al. 2007; Jensen 2009; Briggs and Galanopoulou 2011; Cellot and Cherubini 2013; Khazipov et al. 2015), it has been hypothesized that immaturity in GABAergic signaling leading to neuronal excitation may also be a determining condition for seizure activity and epilepsies at other ages (Muñoz et al. 2007; Khazipov et al. 2015; Löscher et al. 2020; Liu et al. 2020).

On the other hand, an excessive neuronal excitation mediated by amino acids leads to neuronal death through a process known as excitotoxicity (Dodd 2002; Babot et al. 2005; Dong et al. 2009; Zhao et al. 2011). Thus, during seizure activity, increased extracellular levels of glutamate and GABA can lead to both excessive neuronal excitation and seizure-mediated excitotoxic neuronal death. In addition, the neuronal loss by whatever degenerative process in specific areas of the brain may induce seizures (Fujikawa 2005; Vincent and Mulle 2009; Chen et al. 2010; Niquet et al. 2012). Then, the relationship between seizures and excitotoxicity is closely reciprocal, and the control of one any of them could lead to control of both.

12.2 Glutamate-Mediated Excitotoxicity and Neuronal Death in Neurological Illnesses

The term “excitotoxicity” was coined by J.W. Olney to refer to neuronal death caused by overactivation of glutamate-sensitive receptors (Olney et al. 1971). This kind of death was observed for the first time, during the experimental application of monosodium glutamate (MSG) in high concentrations to treat the retinal atrophy, increasing the neuronal excitation (Lucas and Newhouse 1957). Subsequently, glutamate-mediated excitotoxicity was observed in several regions of the brain and was also related to the overexpression of glutamate receptors (Olney 1971; Garattini 1979; Choi and Rothman 1990; Young et al. 2004; Choi 2020). Now the term is applied to the neuronal death produced by a neuronal sustained excitation triggered by the overactivation of the glutamate receptors or by other mechanisms, among

which the GABA receptors overactivation may be implicated, particularly when their activation leads to neuronal excitation (Nuñez et al. 2003; Zhao et al. 2011). However, the excitotoxicity triggered by glutamate, or its analogs, is the best-known excitotoxic process, and it has been extensively associated with the neuronal death observed in several neuropathological conditions, including epilepsy (Lipton and Rosenberg 1994; Caudle and Zhang 2009; Dong et al. 2009; Choi 2020).

12.2.1 *Glutamate Receptors*

The excitatory glutamate effects depend on its specific interaction with cell membrane receptors, functionally classified as ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors, which act as ligand-gated ion channels or as G protein-coupled receptors, respectively (Hassel and Dingledine 2006). In general, the ionotropic glutamate receptors mediate the neuronal fast depolarization allowing the Na^+ and Ca^{2+} influx and the K^+ efflux through the same ionic pore; and they are classified according to their affinity to specific exogenous agonists in sensitive receptors to N-methyl-D-aspartate (NMDAR), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainic acid (KAR) (Watkins and Olverman 1987; Kohl and Dannhardt 2001; Simeone et al. 2004; Vincent and Mulle 2009; Hansen et al. 2021). Structurally, they are oligomeric macromolecular complexes formed classically by four polypeptide subunits, each of which contains an amino-terminal extracellular domain, followed by a transmembrane domain (TM1), a loop partially embedded in the membrane cytosolic face (TM2), other two transmembrane domains (TM3-4) and the carboxy-terminal intracellular domain (Simeone et al. 2004; Vandenberghe and Brecht 2004; Flores-Soto et al. 2013). The endogenous ligand glutamate interacts specifically in the neighborhood between the amino-terminal loop and the extracellular spacer loop of TM3 and TM4 (Wollmuth and Sobolevsky 2004; Flores et al. 2013) (Fig. 12.1). For each type of iGluR there are several families of subunits that, differentially distributed, may originate receptors functionally different, but activated for the same endogenous ligand, glutamate (Holopainen and Laurén 2012; Simeone et al. 2004; Wollmuth and Sobolevsky 2004). Additionally, by sequence homology, a fourth iGluR type called the GluD (or delta) receptor has been identified, but its specific endogenous ligand and whether it forms functional ion channels remains to be established (Hansen et al. 2021).

The NMDAR is characterized by its voltage dependency and high permeability to Ca^{2+} , which according to its composition in subunits, could be slowly or rapidly inactivated (Popescu and Auerbach 2003; Simeone et al. 2004; Hansen et al. 2021). It has multiple pharmacological regulatory sites, described as binding sites for (1) L-glutamate as a transmitter or endogenous ligand, and also for its competitive agonists and antagonists; (2) glycine or D-serine as coagonists; (3) phencyclidine and dizocilpine (MK801) as channel blockers; (4) Mg^{2+} as channel blocker that can be removed by depolarization; (5) Zn^{2+} as positive modulator; (6) polyamines as positive or negative modulators, depending on the compound and their concentration;

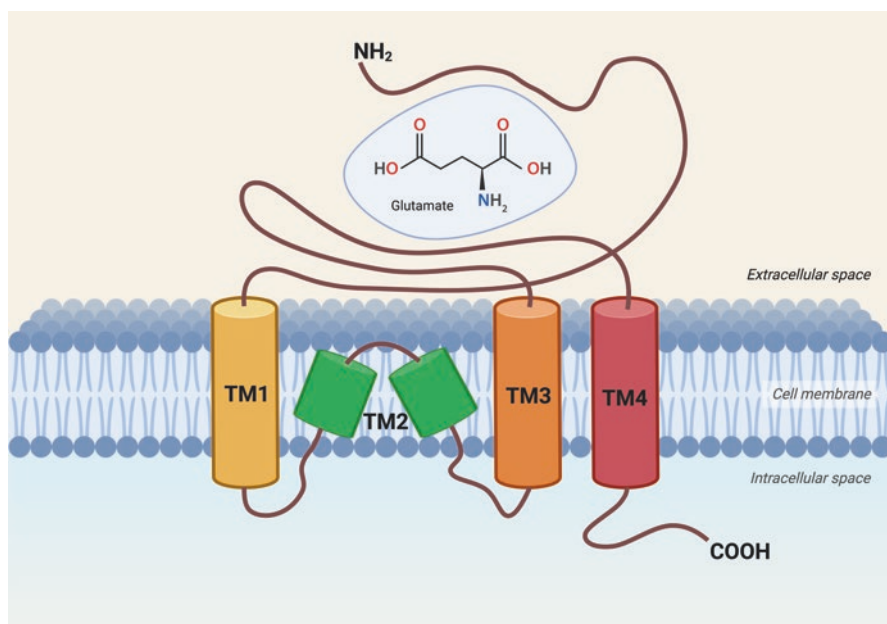


Fig. 12.1 Conformational distribution of transmembrane domains and extracellular and intracellular loops of ionotropic glutamate receptors subunits showed schematically. The extracellular loops build the binding site for glutamate, which may be exchanged by the glutamate agonist analogs in non-NMDAR and by glycine in the NR1 and D-serine in the NR3 subunits of NMDAR

and (7) a site sensitive to redox changes. Structurally, this receptor is an obligated heterotetramer conformed by combinations of the NR1 subunit (existing in eight edition variants) with the NR2A-D, NR3A-B, or both subunits, where the presence of NR1 determines the existence of a functional ion channel, while NR2A-D and NR3A-B modify the electrophysiological properties of the channel. Because each subunit family is sensitive to different agonists, NR1 to glycine, NR2 to glutamate, and NR3 to D-serine, NMDAR activation requires more than one agonist to be activated (Popescu and Auerbach 2003; Simeone et al. 2004; Wollmuth and Sobolevsky 2004; Holopainen and Laurén 2012; Flores-Soto et al. 2013; Hansen et al. 2021) (Fig. 12.2).

Non-NMDA receptors (AMPA and KAR) are membrane voltage-independent, highly permeable to Na⁺, and respond to glutamate faster than NMDARs, with which they coexist on most postsynaptic membranes (Holopainen and Laurén 2012; Hansen et al. 2021). The AMPAR also recognizes kainic acid but with low affinity compared to KAR. It is conformed as homomeric or heteromeric tetramer from the GluR1-4 subunits, whose mRNA splice variants, and in particular Q/R site editing, can change ligand selectivity as well as channel permeability and kinetic properties, leading to Ca²⁺ influx (Bettler and Mulle 1995; Simeone et al. 2004; Vandenberghe and Brecht 2004; Vincent and Mulle 2009). Besides, homomeric and heteromeric

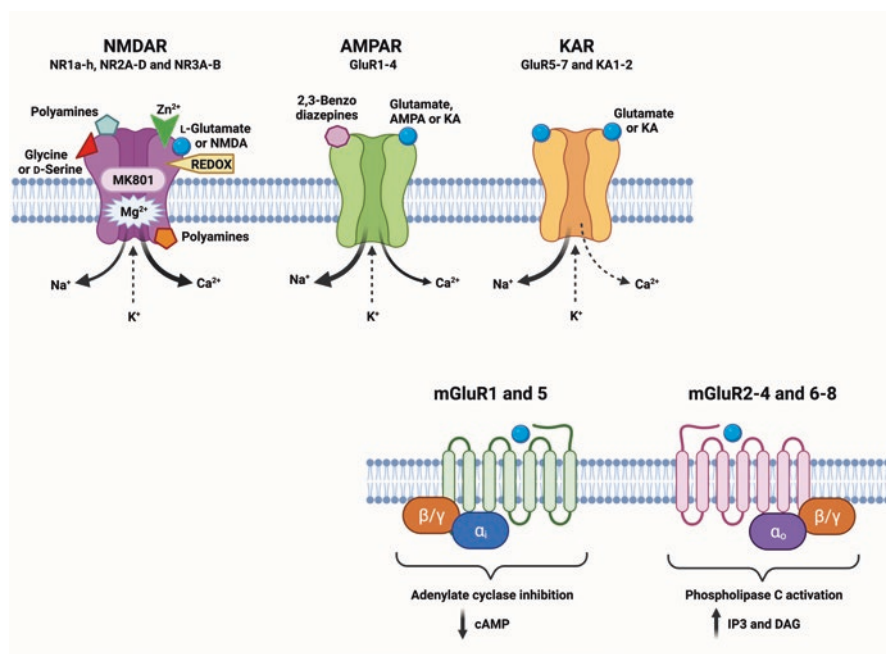


Fig. 12.2 Pharmacological binding sites, conformational subunits, and responses of the glutamate receptors showed schematically. The intensity and continuity of arrows are associated with the amplitude of the ionic currents triggered through each ionotropic glutamate receptors when they are activated for their agonists (upper panel). In the metabotropic glutamate receptors, different intracellular messengers are activated for each subtype (bottom panel)

tetramers of GluR5-7 with KA1-2 proteins build KARs, which show a high affinity by kainic acid being predominantly permeable at Na⁺ (Bettler and Mulle 1995; Vincent and Mulle 2009) (Fig. 12.2).

On the other hand, mGluRs exist in homo- or heterodimeric associations, where each polypeptide contains seven helical segments that wrap back and forth through the membrane, with the extracellular amino-terminal and the intracellular carboxyl-terminal domains unusually large in comparison with other metabotropic receptors (Kunishima et al. 2000; Simeone et al. 2004; Holopainen and Laurén 2012; Stansley and Conn 2019). Eight different mGluRs identified in the nervous system have been subdivided into three groups based on their sequence homologies and enzymatic coupling. mGluR1 and mGluR5 of group I, activate a G protein-coupled to phospholipase C activation and IP3 and DAG generation, while mGluR2-3 of group II and mGluR4,6-8 of group III inhibit the production of cAMP by inhibitory G protein activation (Kunishima et al. 2000; Holopainen and Laurén 2012) (Fig. 12.2). mGluR of the groups I and II have extrasynaptic location while group III are predominantly presynaptic, and it is generally accepted that the group I increases the neuronal excitability through inhibition of several K⁺ channels, while those of

Group II and III decrease the release of neurotransmitters such as GABA and glutamate (Kunishima et al. 2000; Simeone et al. 2004; Holopainen and Laurén 2012; Stansley and Conn 2019).

Finally, the synaptic effects mediated by glutamate may also be endogenously exerted by L-aspartate, another dicarboxylic nonessential amino acid, virtually ubiquitous in the human body, but highly concentrated in the brain, and generated as an intermediary metabolite or as a neurotransmitter in different metabolic pools (Hassel and Dingledine 2006; Deutch and Roth 2008; Hansen et al. 2021).

12.2.2 Mechanisms Implicated in the Neuronal Death Produced by Glutamate

Initially, glutamate receptors activation depolarizes the plasma membrane through the influx of ions promoted by their activation. But when the activation is sustained over time, the osmotic imbalance caused by the massive influx of Na^+ and Cl^- leads to cytoplasmatic Ca^{2+} overload (Young et al. 2004; Caudle and Zhang 2009; Dong et al. 2009; Szydlowska and Tymianski 2010; Choi 2020). The influx of Na^+ alters the functionality of cotransporters, pumps, and channels that depend on its electrochemical gradient (Greene and Greenamyre 1996; Dong et al. 2009; Morrison et al. 2013). The influx of Cl^- alters several plasmatic transporters and promotes Ca^{2+} -independent glutamate release that potentiates excitotoxicity (Young et al. 2004; Babot et al. 2005; Zhao et al. 2011; Choi 2020). The cytoplasmatic Ca^{2+} overload can promote: (1) the synthesis of nitric oxide, which could reach the presynaptic glutamatergic terminal to stimulate additional glutamate release through a cGMP-dependent mechanism; (2) the generation of free radicals, such as superoxide or peroxynitrites, which promote lipid peroxidation and destabilization of cell membranes; and (3) the loss of electrochemical mitochondrial potential, that alters the oxidative phosphorylation and promotes the generation of free radicals to the point of completely invalidating mitochondrial energy metabolism. In addition, Ca^{2+} can activate various intracellular signaling pathways dependent on protein kinases and phosphatases that could promote the proteolysis of cell content (Greene and Greenamyre 1996; Montal 1998; Arundine and Tymianski 2003; Young et al. 2004; Dong et al. 2009; Szydlowska and Tymianski 2010; Choi 2020) (Fig. 12.3).

The glutamate-mediated excitotoxicity as a continuous process may be strongly acute in its initial phase and trigger neuronal death by necrosis, but it can also evolve more slowly and cause apoptosis (Young et al. 2004). In this sense, in vitro studies have shown that glutamate can produce both types of death depending on its application scheme (Bonfoco et al. 1995; Portera-Cailliau et al. 1997a, b). Thus, a brief exposition to high concentrations of glutamate could cause acute neuronal death due to early degenerative changes related to tissue inflammatory process that is characterized by being dependent Na^+ and Cl^- . Otherwise, prolonged exposure to lower concentrations of glutamate could lead to delayed neuronal death, which is

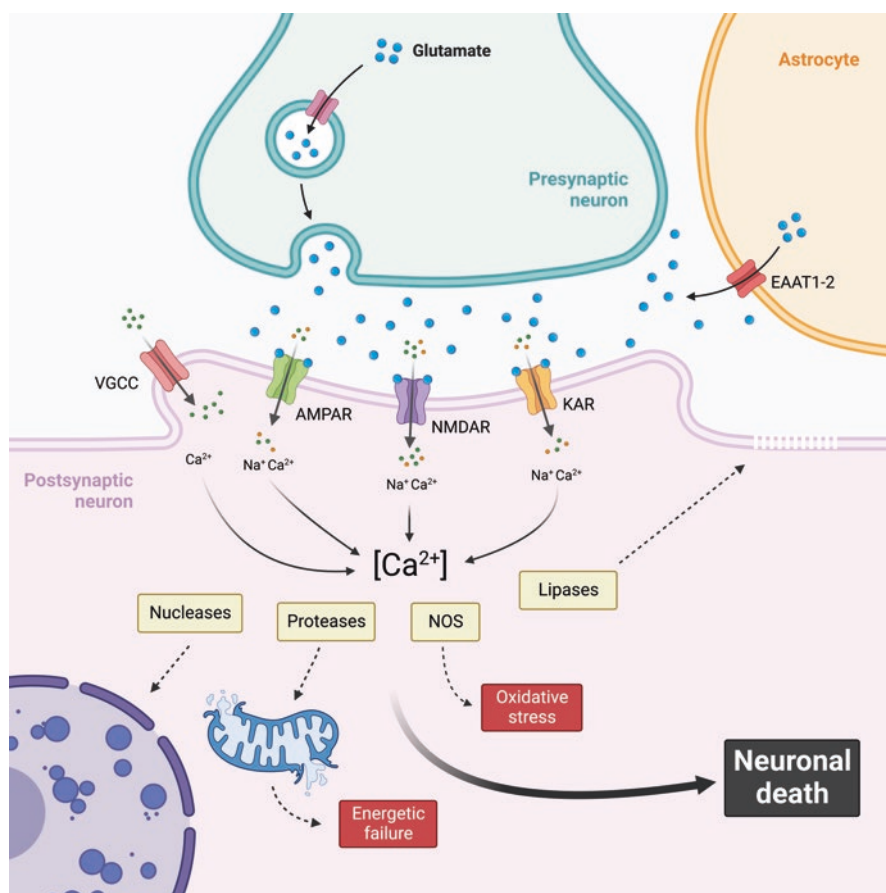


Fig. 12.3 Schematic representation of the most relevant events that lead to glutamate-induced neuronal death, involving ionic imbalance, energy failure, oxidative stress, and intracellular signaling pathways triggered by calcium overload, among other mechanisms. Death can occur by necrosis or apoptosis depending on the triggered stimulus or the initial energetic functional state. Abbreviations: VGCC voltage-gated calcium channels, NOS nitric oxide synthase, EAAT1-2 excitatory amino acid transporters 1 and 2 types

dependent on Ca^{2+} influx and requires several hours or even days to occur (Bonfoco et al. 1995; Portera-Cailliau et al. 1997a, b; Young et al. 2004). Also, it has been suggested that the glutamate-mediated degenerative process depends largely on the functional mitochondrial state and that when the cellular metabolic rate is reduced, the mitochondria is unable to maintain homeostasis of Ca^{2+} and therefore neuronal death is mainly due to apoptosis (Bonfoco et al. 1995; Portera-Cailliau et al. 1997a, b; Young et al. 2004; Niizuma et al. 2010).

12.2.3 Glutamate-Mediated Excitotoxicity and Neurological Illnesses

Studies carried out in different neural systems, both in vivo and in vitro, on glutamate-mediated excitotoxic degeneration have demonstrated that in pathological conditions, such as cerebral hypoxia-ischemia (Choi and Rothman 1990; Szydlowska and Tymianski 2010; Choi 2020), traumatic brain injury (Bramlett and Dietrich 2004; Wagner et al. 2005), epilepsy (Meldrum 1993a; Wilson et al. 1996; Friedman et al. 2003; Sarlo and Holton 2021) and domoic acid (Meldrum 1993b; Jeffery et al. 2004), glutamate concentration increases significantly in the brain, and these increases are closely related to the observed neuronal damage. Additionally, it has been proposed that excitotoxicity participates in the establishment of several neurodegenerative diseases such as Huntington's (Beal et al. 1991; Gardian and Vecsei 2004), Alzheimer's (Ferrarese et al. 2000; Hynd et al. 2004) and Parkinson's diseases (Lipton and Rosenberg 1994; Rego and Oliveira 2003; Caudle and Zhang 2009), as well as in schizophrenia (Lipton and Rosenberg 1994), among other degenerative processes. In this regard, experimental trials have shown that glutamate antagonists could protect against neuronal excitotoxic damage and control seizures, reducing neurodegenerative processes (Meldrum 1985; Morales-Villagran et al. 1996; Harty and Rogawski 2000; Löscher et al. 2020). In clinical trials, this knowledge has been applied with some success; for example, memantine, one of the therapeutic agents used recently for Alzheimer's disease, although it does not cure the disease, can slow down its progression, acting as NMDAR antagonist (Moreira et al. 2006; Supnet and Bezprozvanny 2010). Also, memantine resembles to exert positive effects on vascular dementia and Parkinson's disease (Olivares et al. 2012). Another example is dizocilpine, one NMDAR ion channel blocker that, applied in combination with nimodipine, appears to decrease the penumbra area in acute excitotoxic neuronal damage caused by a hypoxic-ischemic event, but its neuroprotective effect is variable and sometimes insignificant (Niizuma et al. 2010; Szydlowska and Tymianski 2010). In addition, perampanel, topiramate, and felbamate, which act as iGluR antagonists, appear to control some types of epilepsy, particularly focal epilepsies (Celli and Fornai 2021).

12.3 Systemic Administration of Monosodium Glutamate as Excitotoxicity Model

Although various glutamate agonists have been used to trigger excitotoxic neuronal damage, systemic administration of monosodium glutamate (MSG) is probably the best option to study the glutamate-induced neurodegenerative process in an integral manner. Through this model, it has been possible to temporally characterize the neurophysiological alterations and compensatory responses that follow the

excitotoxic insult, and to establish that most mammalian species are susceptible to the toxic effects of glutamate, and that the severity of the induced damage depends on the species, age, and gender (Garattini 1979). Thus, it is now known that the greatest susceptibility to glutamate-mediated excitotoxicity is observed in: (1) newborn male mammals compared to adults, females and other vertebrates (Garattini 1979); (2) in brain regions where the density of glutamate receptors is higher, such as the hippocampus and cerebral cortex (Meldrum 1993b; Beas-Zarate et al. 2002a; Kim et al. 2009), among others; and (3) in neural and nonneural cells that express glutamate receptors, such as GABA neurons (Reeves et al. 1987; Muller et al. 2001; Ureña-Guerrero et al. 2009), microglial cells (Brown and Neher 2010) and Bergmann glial cells (Mendez-Flores et al. 2016), among others.

The immaturity of the blood-brain barrier (Xu and Ling 1994; Ek et al. 2006; Bell et al. 2020), low glutamate uptake (Thomas et al. 2011; Rose et al. 2018), long amplitude and duration of NMDA- and voltage-gated Ca^{2+} currents (Ben-Ari 2001; Jensen 2009; Dehorter et al. 2012), and GABA-mediated excitability (Nuñez et al. 2003; Ben-Ari et al. 2007; Zhao et al. 2011), are some of the conditions associated with the high susceptibility to glutamate-mediated excitotoxicity characteristically observed in newborns. However, systemically administered MSG can also induce damage in adulthood, particularly in areas of the brain where the blood-brain barrier is deficient, such as the arcuate nucleus and other hypothalamic nuclei (Garattini 1979; Hu et al. 1998). Additionally, also it is known that glutamate-mediated excitotoxicity could be associated with seizures (Arauz-Contreras and Feria-Velasco 1984; Lopez-Perez et al. 2010), obesity (Garattini 1979; Hu et al. 1998; Kirk et al. 2009; Hernández Bautista et al. 2014; Andres-Hernando et al. 2021), migraine (Benbow et al. 2022), and learning (Ishikawa et al. 1997; Velazquez-Zamora et al. 2011) and motor impairments (Möykkynen and Korpi 2012; Firgany and Sarhan 2020) with the males being more susceptible than females, probably due to the neuroprotective effect exerted by steroids (Luoma et al. 2011).

12.3.1 Changes Induced by Systemically Administered MSG in Neonatal Rats

As mentioned above, systemic administration of MSG to newborn rodents induces acute neuronal damage and compensatory changes, which can be characterized over time. Thus, among the immediate changes, Hu et al. (1998) showed that a single MSG dose of 0.2 mg/g of body weight administered subcutaneously on postnatal day (PD) 7 in male mice, produced a 17-fold elevation of plasma glutamate levels above the initial value, which was associated with increases in the expression level of NR1 and GluR2/4 subunits, and minor but significant injury in subependymal neurons near the base of the third ventricle. More recently, it was shown via an

enzymatic biosensor implanted in the right lateral ventricle of the brain that MSG 4 mg/g of body weight administered subcutaneously to newborn male rats on PD1 increased the extracellular glutamate levels to values close to 300% above the baseline. Increases in the extracellular glutamate levels were more pronounced when the same dose of MSG was re-administrated at PD3 and PD5, but no increases in PD7 were observed after the fourth administration of the same dose of MSG. These increases in the extracellular brain glutamate levels were associated with electrographic and behavioral epileptiform activities, as well as with rises in total glutamate, glutamine, and GABA contents measured in the hippocampus 24 hours after each MSG administration (Lopez-Perez et al. 2010). In addition, using the administration scheme described above, where 4 mg of MSG per gram of body weight is subcutaneously administered to neonatal male rats four times on PD1, 3, 5, and 7 (a model implemented by our working group), the neuronal death by apoptosis was observed in CA1 and CA3 hippocampal regions, as well as in the cerebral cortex, 24 hours after the last administration (Chaparro-Huerta et al. 2002, 2005; Rivera-Cervantes et al. 2004, 2009). This neuronal loss was also associated with changes in the expression levels of NMDAR and AMPAR subunits (Rivera-Cervantes et al. 2004, 2009) and with increases in the levels of p38 kinase protein and in TNF- α proinflammatory cytokine (Chaparro-Huerta et al. 2002, 2005; Rivera-Cervantes et al. 2004, 2009).

Additionally, after neonatal MSG treatment, the loss of pyramidal (Gonzalez-Burgos et al. 2001; Beas-Zarate et al. 2002a; Velazquez-Zamora et al. 2011), GABA-positive (Ureña-Guerrero et al. 2009) and dopaminergic (Lopez-Perez et al. 2005) neurons has been observed in various brain regions of adult rats. This neuronal loss has been associated with changes in the expression level of non-NMDA and NMDA subunits (Beas-Zarate et al. 2001, 2002b, 2007) and of glutamate transporters (Medina-Ceja et al. 2012; Castañeda-Cabral et al. 2020); in the binding sites to acetylcholine, and choline acetyltransferase activity (Ortuño-Sahagun et al. 1997); as well as in dopamine receptors and transporters (Lopez-Perez et al. 2005); in the [3 H]-GABA release (Beas-Zarate et al. 1998) and uptake (Ureña-Guerrero et al. 2009); in glutamic acid decarboxylase activity (Ureña-Guerrero et al. 2003); and in others GABAergic markers (Ureña-Guerrero et al. 2009); all of them observed in different brain regions and ages after treatment until adulthood. Furthermore, the MSG neonatal treatment induces hyperplasia and hypertrophy on astrocytes and microglial cells in the cerebral cortex and hippocampus of adult rats (Martinez-Contreras et al. 2002; Castañeda-Cabral et al. 2020). In this point, it is important to mention that neonatal MSG treatment produces significant changes in seizure susceptibility (Ureña-Guerrero and Beas-Zarate 2006), as well as in learning capacity (Gonzalez-Burgos et al. 2001; Velazquez-Zamora et al. 2011), both of which are closely related with the modifications described above.

12.4 Changes in Adulthood Seizure Susceptibility After MSG Neonatal Treatment and Its Possible Relationship with the Pharmacoresistance

When we observed that adult rats neonatally treated with MSG developed an unusual wild running behavior after simple manipulations as cage switching, and consistent with the significant changes induced in GABAergic and glutamatergic neurotransmission systems mentioned above, we decided to characterize seizure susceptibility through some experimental models to induce convulsions. First, we used 4-aminopyridine as a generic convulsive drug that acts as a blocker of voltage-sensitive potassium channels; followed by iodide-methyl-bicuculline as GABA antagonist and NMDA as glutamate agonist, all of them administered intracerebrally into the right lateral ventricle in awake adult rats (Ureña-Guerrero and Beas-Zarate 2006). Except to NMDA, all convulsive drugs induced more severe convulsive symptoms in the MSG-treated group than in the control group. Moreover, the seizure latency was shorter, and the seizure duration was longer in the MSG-treated group than in the control group (Ureña-Guerrero and Beas-Zarate 2006; Hernandez-Ojeda et al. 2017) (Table 12.1). Intracerebroventricular (i.c.v.) administration of NMDA (10 nmol) in the MSG-treated group produced repenting, intense jumps and tremors, and facial clonus and forelimb clonus. Still, the motor behavioral alterations disappeared during the first 15 minutes. They did not generate any epileptiform

Table 12.1 Minimal dose of some convulsive drugs necessary to induce severe convulsive signs in adulthood after MSG neonatal treatment

	Convulsive drugs (doses)					
	4-Aminopyridine (1, 2, 3, 4, and 5 nmol)		Iodide-methyl bicuculline (0.25, 0.5, 1, 1.5, and 2 nmol)		N-Methyl-D- Aspartate (2.5, 5, 7.5, and 10 nmol)	
Severe convulsive signs	Control	MSG- treated	Control	MSG- treated	Control	MSG- treated
Wild running	3.0	1.0	1.5	0.5	–	–
Rearing	2.0	1.0	1.0	0.25	5.0	–
Generalized tonic–clonic convulsions	4.0	2.0	2.0	1.0	5.0	–
Status epilepticus establishment	4.0	2.0	–	1.0	7.5	–
Animal death (%) ^a	4.0 (25%)	2.0 (80%)	–	1.5 (37.5%)	10 (50%)	–

Data indicate the dose at which each severe convulsive sign was observed in each experimental group. Doses tested are indicated in parentheses under each convulsive drug

^aThe percentage of animal death was estimated from eight animals for each group, convulsive drug, and dose

discharge in the hippocampus of adult rats, while in the control group, behavioral and electrographically, the NMDA injection induced generalized tonic-clonic convulsions, status epilepticus, and death (Ureña-Guerrero and Beas Zarate 2006) (Table 12.1). Interestingly, electrographic recordings of basal activity in the hippocampus and entorhinal cortex of MSG-treated adult rats were characterized by a lower net amplitude and higher average firing frequency than that observed in the control group. In addition, 3 nmol of 4-aminopyridine via i.c.v. induced a greater number of firing bursts with higher net amplitude and longer duration in MSG-treated adult rats (Hernández-Ojeda et al. 2017).

Thus, although more studies are necessary, the evidence suggests that after neonatal MSG treatment, some adaptive changes occur at the level of NMDA receptors that could generate some type of resistance to NMDA agonists. In this sense, it is important to mention that when neonatal MSG treatment is administered to male rats, the NMDAR is more abundant than the non-NMDAR (Simeone et al. 2004; Holopainen and Laurén 2012), particularly in the cerebral cortex and the hippocampus, where any electrographic epileptiform discharges were recorded after intracerebral NMDA administration in adult rats treated with MSG. In addition, experimental evidence has been demonstrated that NMDAR activation could lead to its structural and functional modification resembling any kind of “habituation ligand-receptor” or “preconditioning”, where the NMDAR does not become responsive to NMDA (Boeck et al. 2004; Severino et al. 2011). Then, neonatal MSG treatment could induce a pronounced preconditioning, which seems to remain until adulthood, where NMDA i.c.v. administration does not induce the epileptiform activity observed in control rats (Ureña-Guerrero and Beas-Zarate 2006). In this sense, the NMDAR functional modifications have also been suggested in the studies where learning impairment has been reported after neonatal MSG treatment (Gonzalez-Burgos et al. 2001; Velazquez-Zamora et al. 2011). According to the last, although the pharmacoresistance in epilepsy has been primarily related to changes in the expression levels of voltage-gated sodium and calcium channels, GABA_A receptor subunits, and efflux transporters (Remy and Beck 2006; Löscher et al. 2020), it is possible that MSG neonatal treatment may induce some form of pharmacoresistance, especially for anticonvulsive drugs that act on NMDAR (Celli and Fornai 2021), such as felbamate (Harty and Rogawski 2000) and lamotrigine (Wang et al. 1996). In this sense, it has been reported that a short preconditioning with NMDA is able to diminish the anticonvulsive efficacy of lamotrigine, without a significant effect on felbamate (Tomczyk et al. 2007).

Finally, the changes induced in non-NMDA receptors after MSG treatment (Rivera-Cervantes et al. 2004, 2009; Beas-Zarate et al. 2007) could be involved in seizure susceptibility, but also, they could be originating some form of pharmacoresistance for the drugs acting through those receptors (Lasoñ et al. 2011; Celli and Fornai 2021). Then, the changes induced by neonatal MSG treatment on glutamate receptors remain to be deeply characterized, particularly their association with a possible pharmacoresistance to NMDA.

12.5 Concluding Remarks and Perspectives

Because the increases in extracellular brain glutamate levels induced by neonatal MSG treatment resemble those seen in various neonatal neurological disorders, including hypoxic-ischemic and anoxic episodes, traumatic brain injury, and seizures, an in-depth characterization of treatment-induced changes in the brain is important to elucidate the mechanisms associated with both seizure susceptibility and drug resistance observed in humans after excitotoxic damage. We considered the pharmacological and electrophysiological characterization of glutamate and GABA receptors to be particularly important after neonatal MSG treatment, as they have been deeply implicated in seizure susceptibility and excitotoxicity, as well as in pharmacoresistance in epilepsy.

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Chapter 13

Cerebrovascular Remodeling and the Role of Vascular Endothelial Growth Factor in the Epileptic Brain and Pharmacoresistance



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Abstract The neurovascular unit plays a relevant role in normal situations and pathological conditions involving the nervous system. In epileptic conditions, cerebrovascular remodeling is a dynamic process affected by the activation of several cells, genes, receptors, cytokines, chemokines, and growth factors. Vascular dysfunction, blood-brain barrier (BBB) disruption, and aberrant angiogenesis mediated by vascular endothelial growth factor (VEGF) signaling pathways have been proposed to contribute to epileptogenesis. In the epileptic brain, BBB disruption induces seizures. In addition, excessive angiogenesis may contribute to BBB dysfunction. It has been suggested that BBB dysfunction triggers drug-resistant epilepsy. Evidence indicates that VEGF-related proteins are upregulated in experimental and human epilepsy, suggesting a critical role in the epileptogenesis of this cytokine. VEGF may play a neuroprotective role following seizures but also can induce BBB leakage and pathological angiogenesis, suggesting a dual effect in epileptic tissue. Therefore, cerebrovascular remodeling and VEGF signaling could be therapeutic targets in pharmacoresistant epilepsy.

Keywords Drug-resistant epilepsy · Vascular dysfunction · Blood-brain barrier · Angiogenesis · Vascular endothelial growth factor

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13.1 Introduction

Although the pathophysiology of epilepsy is a complex process, vascular anomalies have become important in the process of epileptogenesis. In addition, irreversible changes to neuronal networks persisting after inflammatory responses and vascular dysfunction may lead to spontaneous seizure occurrence (Tan et al. 2021). Damage to the blood-brain barrier (BBB) has been observed in experimental models of epilepsy, as well as in tissue from patients with drug-resistant epilepsy (DRE) (van Vliet et al. 2007; Marchi and Lerner-Natoli 2013). Evidence supports the hypothesis that cerebrovascular remodeling could contribute to epileptogenesis and the propagation of epileptic seizures (van Vliet et al. 2007; Morin-Brureau et al. 2012; Baruah et al. 2020). BBB dysfunction may contribute to the development of epilepsy after status epilepticus (SE) either via a neuronal depolarization by the influx of K^+ or a via a cascade of events triggered by BBB leakage that leads to glial activation, impaired K^+ buffering, an increase of blood pressure, dysregulation of cerebral blood flow, oxidative stress, angiogenesis, and inflammation (Gorter et al. 2015). Because pericytes are involved in angiogenesis, the alteration of pericyte-astroglial cell interactions in epileptic conditions might contribute to aberrant angiogenesis (Kovács et al. 2012; Klement et al. 2018). Therefore, cerebrovascular remodeling could be a therapeutic target in DRE.

The cerebrovascular network consists of the interface and association between the brain and blood vessels. However, to achieve the correct functioning of this cerebrovascular network, the participation and communication of glial cells, pericytes, and neurons are required. The result of this interaction is known as the neurovascular unit (NVU) (Swissa et al. 2019). The NVU represents a structural and functional interaction between the cerebral parenchyma and blood circulation. The NVU also includes pericytes that cover a large part of endothelial cells, and astrocytic endfeet, that surround the vessels on the brain parenchyma side (Langen et al. 2019), as illustrated in Fig. 13.1. This unit generates homeostasis and regulation of cerebral blood flow (Iadecola 2017). Astrocytes provide lactate and oxygen to support neuronal homeostasis. Pericytes have cytoskeleton systems that are associated with cell contraction, which enables the regulation of vessel diameters and changes in blood flow that could affect neuronal activity. Endothelial cells release vasoactive factors to regulate vessel diameters, possibly resulting in changes in neuronal activity (Hawkins and Davis 2005; Ogaki et al. 2020). Failure of the BBB is a critical event in the development and progression of several diseases that affect the central nervous system (CNS). In some neurological conditions, increased BBB permeability is a consequence of the pathology, whereas in others, BBB opening may be a precipitating event (Hawkins and Davis 2005). However, the main factor in cerebrovascular diseases is the breakdown of the BBB, which is characterized by the infiltration of blood components, abnormal transport, and the leakage of molecules into the CNS (Rigau et al. 2007; Morin-Brureau et al. 2011). NVU remodeling is rapidly activated after seizures and occurs at the cellular and molecular levels. After seizures, proangiogenic genes and growth factors are

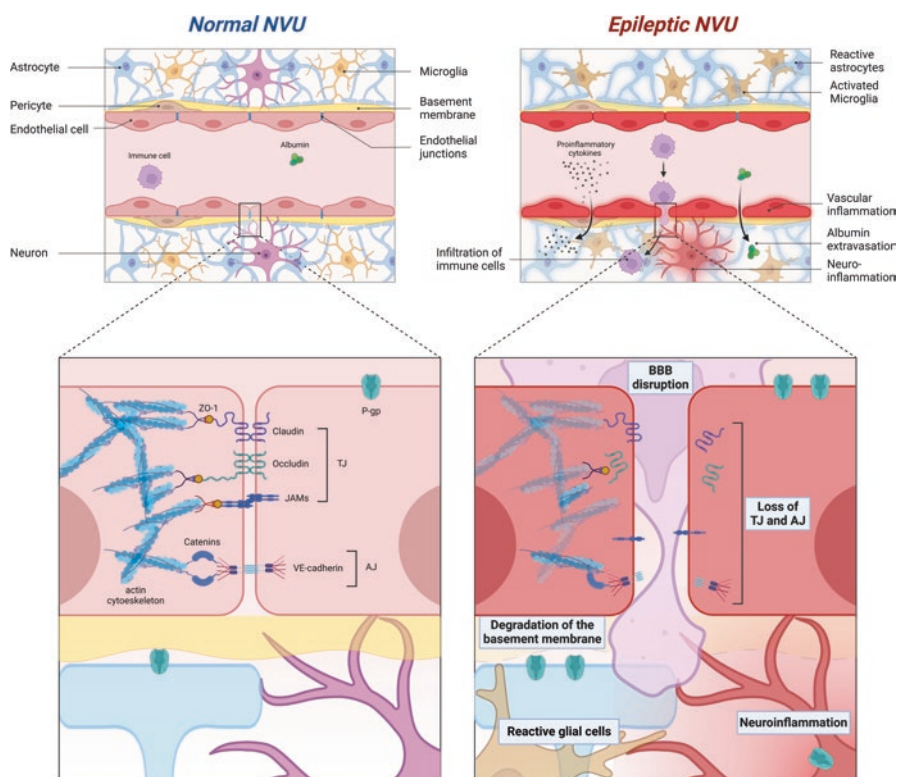


Fig. 13.1 Neurovascular unit (NVU) in the normal and epileptic brain. The NVU consists of astrocytic endfeet, pericytes, endothelial cells, and neurons. The basement membrane also is present between endothelial cells and astrocytes. Pericytes are inserted in the vascular basement membrane. In the normal NVU, astrocytes provide support for neuronal homeostasis. Pericytes regulate vessel diameters and blood flow. Endothelial cells are communicated by endothelial junctions including adherens junctions (AJ) and tight junctions (TJ). The main proteins that integrate the AJ are vascular endothelial-cadherin (VE-cadherin) and catenin. TJ is formed by claudin, occludin, zonula occludens 1 (ZO-1), and junctional adhesion molecule (JAM) proteins. At normal NVU, the P-glycoprotein (P-gp) is localized in the endothelial cells and astrocytes. In the epileptic NVU, there exist reactive glial cells (astrocytes and microglia) that release proinflammatory cytokines, which generate vascular and neuronal inflammation. In addition, in the epileptic NVU, expression of TJ and AJ proteins is downregulated, which induces blood-brain barrier (BBB) disruption. This disruption promotes the infiltration of immune cells and albumin extravasation, which generates an inflammatory environment at NVU contributing to the remodeling of neuronal and vascular networks. The basement membrane is degraded in the epileptic NVU. Pharmacoresistant epilepsy is associated with the overexpression of P-gp on endothelial and astrocytic cells. Neuronal P-gp expression is related to increased cellular excitability and plays a central role in the epileptogenic process

upregulated to promote angiogenesis and survival of glial and neuronal cells (Freitas-Andrade et al. 2020). BBB disruption and excessive angiogenesis are features observed in tissue from epilepsy patients and in experimental models of epilepsy (Marchi and Lerner-Natoli 2013). This excessive and aberrant angiogenesis is

primarily promoted by vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 (Morin-Brureau et al. 2011; Baruah et al. 2020). In addition, excessive VEGF expression induces BBB breakdown and vascular leakage (Carmeliet and Ruiz de Almodovar 2013). VEGF is a factor that interacts between the nervous system and the vascular system. Because VEGF has effects on both cerebral vasculature and nervous tissue, the role of this factor in epilepsy is crucial and similarly might represent a crucial in the development of pharmacoresistant epilepsy.

13.2 Vascular Remodeling

Vascular remodeling is crucial in maintaining the homeostasis of blood vessels that respond to their constantly changing environment. Cerebrovascular remodeling is a dynamic process affected by the activation of several cells, genes, receptors, cytokines, chemokines, and growth factors. However, vascular remodeling can also become pathological, where hypoxia plays a significant role in both physiological and pathological remodeling (Silpanisong and Pearce 2013). Hypoxia is considered a primary switch to induce the expression of angiogenic factors. When cells suffer hypoxia, they release angiogenic factors to reestablish oxygen supply through vessel formation. The principal angiogenesis mechanism is the transcription of hypoxia-inducible factors (HIFs), HIF-1 and HIF-2, which can induce transcription of VEGF. Hypoxia also increases the half-life of VEGF mRNA by promoting its stabilization. However, under conditions of low oxygen and nutrients, the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) also promotes VEGF expression, independently of the canonical HIF pathway (Ruiz de Almodovar et al. 2009). HIF-1 α is a prominent activator of VEGF gene expression and inflammatory cytokines, which also regulate VEGF expression. Interestingly, it has been shown that the expression levels of HIF-1 α and tumoral necrosis factor alpha (TNF- α) are overexpressed in tissue dissected of mesial DRE patients, suggesting that these factors contribute to the pathogenesis of this neurological disorder (Gong et al. 2018; Li et al. 2018). Chronic hypoxia stimulates angiogenesis, increases capillary density, and reduces inter-capillary distances within the brain (Silpanisong and Pearce 2013). Since VEGF is expressed by both neuronal and endothelial cells, VEGF-mediated signaling can promote mechanisms that can lead to astroglial activation and precipitate events associated with epilepsy (Baruah et al. 2020). VEGF could activate the VEGFR-2/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS) pathway to induce angiogenesis and vasodilation after hypoxia/ischemia, leading to increase cerebral blood flow. Activation of the VEGF/VEGFR-2/Src signaling pathway promotes vasopermeability through the internalization and subsequent disruption of intercellular junctions of the BBB (Geiseler and Morland 2018). PI3K is involved in cell survival, protein kinase C (PKC) in vascularization, and Src in zonula occludens-1 (ZO-1) downregulation. Seizures promote BBB dysfunction through VEGF/VEGFR-2 signaling pathway (Morin-Brureau et al. 2011). On the other

hand, BBB permeability induces seizures. Neurons have been shown to exhibit epileptiform discharges induced by serum that is leaked from the blood flow. In addition, in the chronic epileptic phase, angiogenesis may contribute to BBB breakdown. It has been suggested that BBB dysfunction triggers drug resistance in epilepsy (Rigau et al. 2007; Morin-Brureau et al. 2012; Ogaki et al. 2020). This loop between vascular dysfunction and continuous seizures could be a relevant target for the development of new therapeutic strategies in DRE.

Inflammation in both neuronal and vascular cells has been proposed as the main mechanism of damage to the BBB (Baruah et al. 2020). This inflammatory response involves the synthesis and release of proinflammatory cytokines and chemokines from glial cells, neurons, and endothelial cells altering the transport mechanisms of BBB and consequently increasing the extravasation of molecules into the brain parenchyma (van Vliet et al. 2014; Herrera and González-Candia 2021). Pericytes exhibit flexible roles as well as regulation of neuroinflammation. Their functions include their ability to express inflammatory cytokines, respond to inflammatory mediators, and participate in neutrophil recruitment (Thurgur and Pinteaux 2019). Pericyte-microglia clustering was observed in the epileptic brain contributing to BBB dysfunction (Klement et al. 2018; Löscher and Friedman 2020). Pericytes are recruited from the periphery and are implicated in blood vessel stabilization during angiogenesis (Thurgur and Pinteaux 2019). Reactive glial cells contribute to the BBB breakdown by downregulation of claudin-5, occludin, and ZO-1 (Herrera and González-Candia 2021). SE induces overexpression of integrins and cell adhesion molecules, which cause BBB disruption via the extravasation of leukocytes (Gorter et al. 2015). Prolonged seizure activity increases systemic blood pressure, decreases blood pH, and promotes vasoconstriction of vessels, leading to BBB disruption (van Vliet et al. 2014). Vascular malformations in the brain compress the brain parenchyma resulting in abnormal structures of neurons and inducing the hyperactivity of neurons, which results in epilepsy (Ogaki et al. 2020). In addition, BBB permeability has been observed in both acute and chronic phases of epilepsy (van Vliet et al. 2007). Increased proinflammatory cytokines such as tumor TNF- α and interleukin-1 β (IL-1 β) were observed in the neocortical microvasculature of the BBB of patients with pharmacoresistant temporal lobe epilepsy (TLE). Interestingly, the authors described that in male patients with pharmacoresistant TLE, IL-1 β , and TNF- α were higher than in female patients, highlighting sex differences in cerebrovascular remodeling in DRE (Castañeda-Cabral et al. 2020a). Antagonists of the IL-1 β pathway resolve BBB breakdown and show antiseizure effects on acute seizure activity after SE (Gorter et al. 2015). Thus, the neuroinflammatory response stimulates TNF- α signaling to cause vascular endothelial (VE)-cadherin internalization, reducing the proteins at intercellular and tight junctions (TJ), which generates an increase in BBB permeability (Herrera and González-Candia 2021) and consequently the accumulation of serum proteins into the brain, which may contribute to increased excitability (van Vliet et al. 2007). Otherwise, pericytes that contribute to BBB function also participate in the cerebrovascular modifications observed in epilepsy, resulting in pericyte-glia clustering that promotes BBB dysfunction (Milesi et al. 2014; Garbelli et al. 2015; Klement et al. 2018). The

structural and functional interaction between neurons and vascular cells provides the brain vulnerability to the damage of NVU that result from continuous seizures. These vascular responses to seizures contribute to neuronal and vascular injury.

13.3 BBB Dysfunction

The BBB is composed of cellular and molecular elements that configure the most relevant function of this structure, namely the selective permeability of molecules and other elements from the bloodstream to the brain parenchyma, thus being vital for the maintenance of brain homeostasis (Correale and Villa 2009; Abbot et al. 2010). The BBB functions primarily as a diffusion barrier for xenobiotics, limiting access to small, lipophilic, and uncharged compounds, including antiseizure drugs (ASDs) (Löscher and Friedman 2020). The restrictive nature of the BBB offers an impediment to drug delivery to the CNS, and significant efforts have been made to improve it, mainly for the delivery of therapeutic drugs (Daneman and Prat 2015). The principal cellular component of the BBB are the endothelial cells that form the internal blood vessel wall (Langen et al. 2019). Endothelial cells are firmly connected through adherens junctions (AJ) and TJ, which are composed of similar cohesive membrane proteins that seal the intercellular space between neighboring cells and control the selectivity of paracellular transport (Correale and Villa 2009; Sweeney et al. 2019). The first major component of BBB-TJ is claudin-5, a protein that belongs to a family of 27 members known to date, which structurally have characteristic four transmembrane domains and that become to build transmembrane tetrameric arrays (Vanlandewijck et al. 2018; Hempel et al. 2020). Recently claudin-25 has also received attention because it establishes structurally important interactions with occludin (Hashimoto et al. 2020). Other members of the claudin family expressed at lower levels in the BBB include claudin-1, -3, -11, -12, and -27 (Haseloff et al. 2015; Berndt et al. 2019), but their role in barrier functions remains unclear. The second major molecular component of BBB are three prototypical proteins from the group collectively referred to as TAMPs (TJ-associated MARVEL-domain proteins): occludin (MARVELD1), tricellulin (MARVELD2), and MARVELD3 (Heinemann and Schuetz 2019). All of them have a claudin-like tetrameric structure, with four transmembrane domains (Yaffe et al. 2012) and, at least, occludin is known to be closely related to the BBB permeability (Pandit et al. 2020; Kim et al. 2020; Saito et al. 2022). The third major component of TJ is members of the immunoglobulins (Igs) superfamily, termed junctional adhesion molecules (JAMs). This kind of protein has two Ig-like domains bonded by a short segment in a U-shape dimeric structure (Heinemann and Schuetz 2019). Additionally, three kinds of plasma membrane transport systems also are part of the BBB: active efflux transporters that belong to the ATP-binding cassette superfamily (ABC transporters) and reduce or limit the transport of xenobiotics and neurotoxins to the brain (Gil-Martins et al. 2020); carrier-mediated transporters (CMT-transporters) that mediate the entry of molecules such as carbohydrates, hormones, amino acids,

among others through positive electrochemical gradients (Correale and Villa 2009; Sweeney et al. 2019); and receptor-mediated transporters where a ligand is specifically recognized to be transported (Kaya and Ahishali 2021). These elements provide a chemical barrier on the endothelial cells of the brain capillaries by actively pumping potentially toxic lipophilic compounds back into the blood, limiting the entry of several drugs into the brain (Löscher and Friedman 2020).

Dysfunctional or structurally damaged BBB appears in various neuropathological processes such as epilepsy, where molecules whose intracerebral concentration is highly regulated (ions, glucose, amino acids, hormones, and albumin) or which should not be in the brain parenchyma (immune cells) are leaked and inducing ion dysregulation, edema, and neuroinflammation (Janigro 2012; Liu et al. 2012; Profaci et al. 2020). Microglial cells surrounding cerebral vessels are involved in maintaining the integrity of the BBB, and vascular activation, BBB breakdown, and microglia activation have been shown to be related (Fig. 13.1). In addition, BBB-microglia interactions have been observed in both acute and chronic neurological disorders (Thurgur and Pinteaux 2019).

During the development, VE-cadherin, a protein that constitutes AJ, promotes the expression of claudin-5 and mediates the interactions between AJ and TJ, contributing to the formation and maturation of the BBB, via Akt activation and inhibition of β -catenin translocation to the nucleus (Tietz and Engelhardt 2015). In addition, TJ proteins are vulnerable to various physiological and pathological conditions, where their expression level, subcellular location, posttranscriptional maturation, or protein interactions can be altered, and then directly affecting BBB permeability (van Vliet et al. 2014). BBB disruption and altered expression of TJ proteins have been observed in patients with postencephalitic or encephalopathic refractory epilepsy (Kimizu et al. 2018), DRE (Hikmat et al. 2018; Rahman and Copeland 2019; Castañeda-Cabral et al. 2020b), as well as in posttraumatic epilepsy, where BBB breakdown was evident shortly after the trauma, and also in patients who survived up to several years after the trauma, with latency seizures no less than 6 months posttrauma (van Vliet et al. 2020). Downregulation of several proteins such as gap junctional proteins, glial excitatory amino acid transporters, and aquaporins were indicative of astroglial activation and BBB dysfunction (Kovács et al. 2012). Luo and colleagues reported that vitexin (flavonoid) reduces seizure susceptibility by inhibiting inflammation and restoring BBB integrity by increasing the expression of TJ proteins (Luo et al. 2018). Also, matrix metalloproteinases (MMPs), zinc-dependent proteases that degrade fibronectin and laminins, can induce BBB disruption. MMP has been suggested to induce BBB dysfunction in the epileptic brain by degradation of TJ proteins in patients with DRE and in experimental epilepsy (Daneman and Prat 2015; Ogaki et al. 2020; Uprety et al. 2021). The expression and activity of MMP-2 and MMP-9 are significantly increased in experimental epilepsy and can induce epileptogenesis following traumatic brain injury (Uprety et al. 2021). Beyond the ionic changes and alteration of TJ proteins following BBB breakdown, leakage of serum proteins to cerebral parenchyma increases the intracranial pressure resulting in cytotoxic edema, which exacerbates the BBB dysfunction (Kovács et al. 2012). Albumin extravasation has been observed

in patients with TLE, cortical dysplasia associated with DRE, tuberous sclerosis complex, gangliogliomas, and vascular malformations (Löscher and Friedman 2020). In the hippocampus of TLE patients, albumin was observed in neurons and perivascular astrocytes (van Vliet et al. 2007). Following extravasation, albumin can be taken up to neurons, astrocytes, and microglial cells resulting in the alteration of buffering of extracellular K^+ and glutamate, which promotes neuronal hyperexcitability and finally induces epileptiform activity (van Vliet et al. 2015; Löscher and Friedman 2020). These findings suggest that compromised BBB and pathological hyperexcitability can affect disease progression in DRE (Upreti et al. 2021). Albumin can induce neuroinflammation via the upregulation of proinflammatory cytokines and the activation of the transforming growth factor beta (TGF- β) signaling pathway. This inflammatory response is an important mechanism that can contribute to epileptogenesis (van Vliet et al. 2015). Instead, blocking TGF- β -mediated signaling prevented the development of hyperexcitability, epileptogenesis, and reduced seizure susceptibility (Kovács et al. 2012; Upreti et al. 2021). IgG uptake is associated with degenerative changes in human TLE and experimental epilepsy, particularly for interneurons and pyramidal neurons. This suggests that the accumulation of albumin or IgG could be a pathogenic mechanism in epileptogenesis and chronic epilepsy (Michalak et al. 2012). However, albumin or IgG uptake in neurons can only take place in neurons that are already damaged (van Vliet et al. 2014). Then it has been suggested that BBB dysfunction triggers drug resistance in epilepsy by albumin leakage, which generates the ineffectiveness of ASDs (Ogaki et al. 2020). Other proteins in the blood such as thrombin and plasminogen, may also have a role during epileptogenesis and BBB dysfunction. Thrombin, a protease involved in blood clotting, can leak into the brain after seizures. Similarly, plasminogen and its activator are increased in resected brain tissue of patients with DRE (van Vliet et al. 2014). Fibrinogen infiltration during BBB leakage induces vascular clustering of microglial cells, whereas inhibiting fibrinogen decreases microglia activation (Thurgur and Pinteaux 2019). These serum proteins may influence the epileptogenic process because they can modify neuronal activity and inflammatory responses, contributing to the remodeling of neuronal and vascular networks (van Vliet et al. 2014). BBB disruption is observed within minutes to hours after SE in the hippocampus, but also in other brain regions such as the entorhinal cortex, piriform cortex, thalamus, amygdala, septum, endopiriform nucleus, and substantia nigra (Michalak et al. 2013; van Vliet et al. 2014). In addition, BBB disruption in epileptogenesis-associated brain regions has been detected as soon as 5 hours after inducing a SE with pilocarpine, persisting even 48 hours after SE onset (Bankstahl et al. 2018; Mendes et al. 2019). A rapid BBB breakdown can be observed between 1 and 2 days post-SE and is related to cerebral edema (Bankstahl et al. 2018). Moreover, it was also observed that after traumatic injury, BBB leakage is more significant during the first 4 days post-SE, and the increased leakage remains until 60 days post-SE (Nnode-Ekane et al. 2010). On the other hand, recurrent seizures induced with 4-aminopyridine also lead to increased BBB permeability shortly after electrographic seizure onset (Prager et al. 2019). Experimental SE induces early loss of vessels associated with BBB leakage

and formation of thrombocyte clots. Vascular injury is followed by angiogenesis which reestablishes vascular length within 2 weeks after SE and increases cerebral blood flow (Ndode-Ekane et al. 2010). On the other hand, infiltration of peripheral leukocytes from the blood into the brain via the adhesion molecules, including intercellular adhesion molecule 1 and vascular adhesion molecule 1, could promote BBB dysfunction and epilepsy (van Vliet et al. 2015). During the latent and chronic epileptic phase, angiogenesis may contribute to increasing BBB permeability by VEGF/VEGFR-2 signaling pathway, which also promotes vascularization and TJ disassembly (van Vliet et al. 2014). Interestingly, a correlation was reported between BBB permeability and vascular density with the frequency and severity of seizures (Rigau et al. 2007; Marchi et al. 2009). These and other findings obtained both in animal models and in human brain tissue indicate a close relationship between epileptic seizures and BBB disruption, although it is not clear whether the latter is a cause or a consequence of the former, and it is still a topic of discussion (Marchi and Lerner-Natoli 2013; Swissa et al. 2019; Löscher and Friedman 2020; van Vliet et al. 2020; Upreti et al. 2021; Löscher 2022). Seizures produce barrier leakage leading to more seizures and consequently promoting the progression and spread of epilepsy. Thus, BBB leakage is both a consequence and a trigger of seizures and epilepsy (Löscher and Friedman 2020). BBB leakage is extensive during the latent period, when seizures are not present, showing that BBB leakage by itself is less likely to cause spontaneous seizures and more probable to contribute to epileptogenesis (Gorter et al. 2015). Recently, Greene and colleagues reported that the deletion of claudin-5 aggravates seizures and BBB breakdown. In addition, the knockdown of claudin-5 leads to spontaneous recurrent seizures, neuroinflammation, and mortality (Greene et al. 2022). This interestingly shows the paradigm that BBB dysfunction through the regulation of claudin-5 can be an inducer of the epileptogenic process and, therefore, a therapeutic target that should be studied further.

Because any drug administered to control epileptic seizures must pass through the BBB, this functional interface has become of interest in the study of DRE. Although damage to BBB increases its permeability, the multiple alterations that are triggered in the functional expression of key components of the BBB, such as multidrug transporters, drug-metabolizing enzymes, and TJ proteins, significantly affect the delivery and activity of ASD in the brain (Swissa et al. 2019). To this respect, overexpression or activity increased of P-glycoprotein (P-gp) and breast cancer resistant protein (BCRP), both members of the ABC transporters superfamily expressed in the BBB, has been largely related with the refractory condition, in accordance with studies carried out in cerebral tissue removed in surgery of, or retrieved postmortem of patients with pharmacoresistant epilepsies and in animal models (Lazarowski et al. 2007; Löscher et al. 2011; Feldmann et al. 2013; Ghosh et al. 2017; Gil-Martins et al. 2020; Langeh et al. 2020; Vazquez and Fagiolino 2022; Fonseca-Barriendos et al. 2022). Under conditions in which the BBB is disturbed, the functional expression of P-gp may be upregulated in brain endothelial cells and perivascular glia (Löscher and Friedman 2020; Fonseca-Barriendos et al. 2022). However, P-gp overexpression is not restricted to the BBB in DRE, and it has also been observed in brain parenchymal cells, where it modifies membrane

excitability (Aronica et al. 2012; Gil-Martins et al. 2020; Fonseca-Barriendos et al. 2022), as illustrated in Fig. 13.1. Recently, it was shown that P-gp expression and localization differ between patients with tumor-related seizures and posttraumatic epilepsy. This difference could explain the epilepsy severity between these two etiologies (Fonseca-Barriendos et al. 2022). Parallel research has provided evidence indicating that P-gp, BCRP, and other efflux receptors could be overexpressed because of upstream activation of inflammatory effectors (Weidner et al. 2018), since soon after the structural alteration of the BBB there is a massive entry of serum albumin into the brain parenchyma, that activates the TGF- β proinflammatory pathway generating neuroinflammation and angiogenesis (van Vliet et al. 2015; Weissberg et al. 2015; Kimizu et al. 2018). Also, there is evidence of the participation of the mechanistic (previously referred to as mammalian) target of rapamycin (mTOR) signaling pathway in the activation of P-gp in DRE (Chi et al. 2017; Casillas-Espinosa et al. 2020; Bojja et al. 2021).

On the other hand, most ASD undergo biotransformation through the action of various isoforms of cytochrome P450 enzymes (Makowska et al. 2021; Vazquez and Fagiolino 2022; Maqbool et al. 2022), which has also been reported overexpressed in the human pharmacoresistant epileptic brain (Ghosh et al. 2017; Ke et al. 2019; Williams et al. 2019). Although the location of these enzymes in the BBB or in the blood-cerebrospinal fluid barrier is still controversial (Wang and Zuo 2018), it is accepted that BBB metabolic enzymes play a role in DRE (van Vliet et al. 2014).

BBB permeability to large molecules such as albumin does not necessarily indicate free diffusion of small molecules or ions. According to the above, analysis of several ASDs in brain tissue, cerebrospinal fluid, and serum of patients with DRE did not indicate any increase of ASD levels in the brain due to BBB breakdown (Löscher and Friedman 2020). Given the challenge represented by the BBB to the entry of ASD into the brain, some strategies have been developed and tested, mainly in animal models. Synthetic nanoparticles able to cross the BBB by transcytosis or endocytosis (Saraiva et al. 2016) have been successfully used in epileptic rats and mice (Yilmaz et al. 2020; Yousfan et al. 2020); direct intracranial drug delivery in areas near the epileptic focus, throughout intraparenchymal, ventricular or transmeningeal route has been realized, with interesting results (Gernet and Feja 2020), despite the fact that there are still many aspects to be resolved for it to be a viable alternative for epilepsy treatment. ABC transporters inhibition of function or expression has also been tested, offering promising results (Leandro et al. 2019). Convection-enhanced delivery (CED) is a drug-delivery technique that uses hydrostatic pressure to distribute a drug-containing fluid by volume flow directly into the interstitial space within a brain region (Löscher and Friedman 2020). Heiss and coworkers reported the application of CED in patients with DRE to investigate the safety and effectiveness of this method (Heiss et al. 2019). Nevertheless, none of these approaches have been validated in human patients, and a lot of research is still needed for their clinical use. It is necessary to highlight that BBB dysfunction indicates a crucial hallmark of seizures and DRE.

13.4 Aberrant Angiogenesis and Barrierogenesis

Vascular remodeling and dysfunction of the BBB are closely related to aberrant angiogenesis, which is the process of new vessel formation temporally and spatially outside the normal developmental pattern. Aberrant angiogenesis was initially described as part of the changes induced by most tumorigenic processes (Ferrara 2002; Croll et al. 2004), but in the brain, like is described above, it can also be caused by disorders such as seizures (Rigau et al. 2007) and is now a recognized component of epileptogenesis (Morin-Brureau et al. 2012; Löscher and Friedman 2020). In the embryonic stage, angiogenesis occurs following the molecular instructions given by the concentration gradients of various growth factors, such as VEGF, angiopoietins, and basic fibroblast growth factor (bFGF), among other signals (Ferrara 2002; Croll et al. 2004; Bueno et al. 2014). In developmental angiogenesis in the brain, the angiogenesis process includes the rising of a perineuronal vascular plexus, which migrates and grows following an endothelial tip cell, and when it stops, then plexus anastomosis and subsequent vessel formation occur. Newly formed vessels are stabilized by interaction with perivascular cells. The VEGF gradient primarily regulates endothelial tip cell migration (da Fonseca et al. 2014). VEGF and bFGF are synthesized and secreted by endothelial cells, as well as cells surrounding vessels, and are overexpressed in response to various inflammatory and damaging brain conditions, which destabilize existing vessels through basal proteolysis of the membrane, disassembly of interendothelial junctions, and induction of endothelial cell proliferation and migration (Weissberg et al. 2011). Therefore, this neoangiogenesis includes pruning and regression of vessels, can be physiological or pathological, is triggered by metabolic demands or pronounced reactive forces, and is primarily mediated by on-off interplays of VEGF and angiotensin-2. Also, the process includes endothelial cell apoptosis, which appears to be driven by factors, cytokines, and chemokines secreted by immune system cells, such as macrophages (Korn and Augustin 2015). Mechanically, blood flow is the hemodynamic force that influences vasoconstriction, and the pruning and regression of vessels, which also are influenced by tissue oxygenation. Among the signaling pathways involved in neoangiogenesis and regulating some other processes involved, VEGF-, FGF-, Notch-, and WNT-mediated signaling pathways have been distinguished as the most significant, but many other molecular players can be involved (Korn and Augustin 2015; Tregub et al. 2022). It is then evident that neoangiogenesis is a complex multifactorial process that is not yet fully understood but is identified as part of brain pathology in several disorders including epilepsy (Morin-Brureau et al. 2012; Löscher and Friedman 2020; Tregub et al. 2022). The formation of new vessels from preexisting vasculature in the brain has significant effects on the permeability of the BBB, for which a new process has been described, barrierogenesis, which refers to the gradual decrease in paracellular and transcellular transport in endothelial cells, which become more selective to form the barrier. Barrierogenesis is primarily dictated by hemodynamic flow and local oxygen concentrations appear not to be involved (Tran et al. 2022), but both VEGF- and

WNT-mediated signaling pathways are involved in barrierogenesis (van Lanen et al. 2021; Tran et al. 2022; Tregub et al. 2022). At this point, it is important to clarify that although VEGF overexpression can exert neuroprotective effects and promote angiogenesis, its long-lasting high levels delay barrierogenesis, contributing to epileptogenesis, among other disorders (van Lanen et al. 2021).

Now, on October 2022, PubMed searching of terms “angiogenesis and VEGF” in conjunction gives 41,729 results out of a total of 135,042 results obtained searching only “angiogenesis” or of the total 92,544 results searching only VEGF, these comparisons show the relevance that VEGF has in general angiogenesis, but searching results decrease significantly if “brain” term is added, giving only 2539 results for “angiogenesis and VEGF and brain”, which indicates that is knowledge in construction from 1992 and that has taken relevance, particularly in the last 20 years. It should be noted that while there is no complete understanding of vascular remodeling, aberrant angiogenesis, or barrierogenesis, the evidence clearly indicates that specific temporal and spatial regulation of excessive VEGF signaling should be part of upcoming therapeutic approaches in epilepsy. Then, in the next section, some relevant evidence related to VEGF signaling in epilepsy will be described.

13.5 VEGF Signaling in Epilepsy

Interestingly, besides the vascular effects, VEGF also acts directly on different cell types. In addition, VEGF has been considered a multifunctional factor for the CNS. At the cerebral level, VEGF promotes survival and restorative effects, neural regeneration, neurogenesis, and vascular remodeling. Expression and secretion of VEGF after neuronal damage have emerged as an important therapeutic target for DRE by modulation of NVU structure and function (Carmeliet and Ruiz de Almodovar 2013; Ogaki et al. 2020; Ureña-Guerrero et al. 2020). VEGF is a neuroprotective factor that reduces damage associated with seizures, but its vascular effects can exacerbate seizures. Therefore, the role of VEGF after seizures could be protective or negative (Croll et al. 2004).

VEGF is the founding member of a family of homodimeric glycoproteins that are structurally related to the platelet-derived growth factor receptor (PDGFR) family, with five Ig-like domains in the extracellular region. The PDGFR family also has a tyrosine kinase domain with a long kinase insert sequence (Shibuya 2013). In mammals, the VEGF family comprises the following members: VEGF or VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF), which regulate vasculogenesis, angiogenesis, and lymphangiogenesis (Shibuya 2013). Each of these VEGF family members is characterized by the presence of eight conserved cysteine residues, which form a typical cysteine-knot structure (Ruiz de Almodovar et al. 2009). VEGF family ligands bind to high-affinity tyrosine kinase receptors VEGFR-1, VEGFR-2, and VEGFR-3. VEGFRs contain seven Ig-like loops (extracellular region) and a split tyrosine kinase domain (intracellular region).

The second and third Ig domains facilitate ligand binding, while the fourth and seventh domains mediate receptor dimerization (Shibuya 2013; Ruiz de Almodovar et al. 2009). VEGF family members also bind to nontyrosine kinase receptors of the neuropilin (NRP) family, NRP1 and NRP2, which are considered to function as co-receptors for the VEGFRs amplifying their phosphorylation and signaling (Ureña-Guerrero et al. 2020). NRPs are single-spanning transmembrane glycoproteins with extracellular domains A and B, which mediate semaphorin binding, while only domain B is necessary for VEGF binding (Ruiz de Almodovar et al. 2009). NRP-1 is mainly restricted to arterial endothelium and lymphatic vessel valves, whereas NRP-2 is predominantly expressed in venous and lymphatic vasculature (Secker and Harvey 2021). VEGF family members bind to its receptors with differential affinity: VEGF-A binds to VEGFR-1 and VEGFR-2, VEGF-B and PlGF bind to VEGFR-1, whereas VEGF-C and VEGF-D bind to VEGFR-3 and VEGFR-2 (with a lower affinity) (Ruiz de Almodovar et al. 2009). VEGF signaling is activated when the ligand induces VEGFR dimerization followed by subunit transactivation through the reciprocal phosphorylation of their tyrosine kinase intracellular domains, which triggers phosphorylation pathways. In general, VEGFR-1 is related to cell survival and angiogenesis regulation, whereas VEGFR-2 is related to migration and angiogenesis, and VEGFR-3 is related to lymphangiogenesis (Secker and Harvey 2021). During early embryogenesis, VEGFR-2 is a positive signal transducer for the angiogenic process, whereas VEGFR-1 is a suppressor of angiogenesis by trapping VEGF and decreasing the proangiogenic signals from VEGFR-2 (Shibuya 2013). Initially, VEGF was described as a factor increasing vascular permeability (Carmeliet and Ruiz de Almodovar 2013). VEGF/VEGFR-2 signaling pathway stimulates eNOS and increases nitric oxide production, promoting a strong vasodilator effect. In addition, VEGFR-2 activates phospholipase C (PLC), which induces the proteolysis of the basement membrane by MMPs, while the activation of PI3K, Akt, and mitogen-activated protein kinase (MAPK) leading to the disruption of intercellular junctions and cellular migration (Morin-Brureau et al. 2012; Shibuya 2013). On the other hand, in the nervous system, VEGFR-1 and VEGFR-2 are associated with neuroprotection (Ureña-Guerrero et al. 2020; Calvo et al. 2022). The signaling mechanisms via which the VEGF mediates neuronal survival appear to depend on the activation of the PI3K/Akt and MAPK pathways, which in turn inhibits the proapoptotic proteins Bad and caspase-9 and activates nuclear factor kappa B upregulating the expression of survival factors including Bcl-2, Bcl-xL, Mc11, c-IAPs, and c-FLIP (Ruiz de Almodovar et al. 2009; Carmeliet and Ruiz de Almodovar 2013). Although VEGF interacts with neuronal cells mainly via VEGFR-2, it also affects glial cells by activating the MAPK/extracellular signal-regulated kinase (ERK) and PI3K/Akt signaling pathways through VEGFR-1 activation (Ruiz de Almodovar et al. 2009), as illustrated in Fig. 13.2.

VEGF protects hippocampal neurons against glutamate-induced excitotoxicity by activating VEGFR-2 and upregulation of the glutamate receptor subunit GluR2, which reduces Ca^{2+} influx (Carmeliet and Ruiz de Almodovar 2013). VEGF expression is regulated by hypoxia or inflammation by several transcription factors

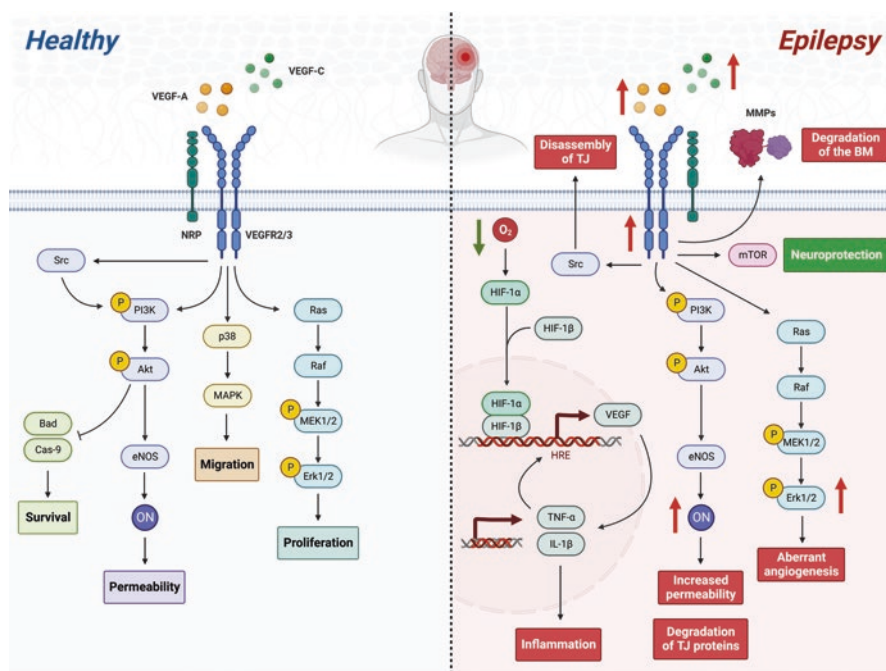


Fig. 13.2 VEGF/VEGFR signaling pathways and functions in the healthy and in epilepsy. In the healthy brain, the binding of VEGF-A or VEGF-C on VEGFR-2, VEGFR-3, and NRP receptors, respectively, induces the activation of signaling pathways such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS) to induce angiogenesis and vasodilation by the production of nitric oxide (NO), leading to increase cerebral blood flow and permeability. In addition, PI3K/Akt signaling pathway inhibits Bad and cas-9 (apoptotic proteins) to induce cell survival. The migration and the proliferation of cells are due to the p38/mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2)/Ras/Raf/MAPK (MEK) activation, respectively. In contrast, in epilepsy, VEGF-A and VEGF-C and their receptors (VEGFR-2, VEGFR-3, and NRP) are overexpressed, leading to increases in the signaling pathways, which subsequently promotes an increase in permeability, degradation of tight junction (TJ) proteins, and aberrant angiogenesis. These pathways activate matrix metalloproteinases (MMPs) involved in the degradation of the basement membrane (BM). VEGF/VEGFR activation also participates in neuroprotection by the mechanistic target of rapamycin (mTOR) activation. Low oxygen (O_2) induces the activation of hypoxia-inducible factors (HIF1 α and HIF1 β) and subsequent translocation to the nucleus and the activation of several genes related to hypoxia. HIF-1 α is a prominent activator of VEGF gene expression and inflammatory cytokines, which also regulate VEGF expression generating an inflammatory response

such as HIF-1 α , AP-1, Sp-1, and signal transducer and activator of transcription 3 (STAT3), which are also activated after seizures (Nicoletti et al. 2008; Morin-Brureau et al. 2012). After seizures, VEGF is overexpressed both in neurons and glia in the hippocampus and cortex. This VEGF increase appears to be cytosolic immunostaining observed in hippocampal and cortex pyramidal neurons (Croll et al. 2004). Enhanced VEGF/VEGFR signaling may attenuate cognitive deficits after epileptic seizures by promoting adult neurogenesis in the hippocampus. VEGF

released from activated microglia in the epileptic brain could contribute to enhanced neurogenesis and neuroprotection via a direct effect (Ogaki et al. 2020). Administration of VEGF on hippocampal slices reduces the amplitude of excitatory responses and suppresses epileptiform activity in experimental epilepsy (McCloskey et al. 2005; Ruiz de Almodovar et al. 2009). Recently, Han and coworkers showed that hippocampal neurogenesis after SE is related to microvascular changes. VEGF exerts protective effects after SE alleviates loss of hippocampal neurons and abnormal vascular regeneration. Upregulation of VEGF expression in the acute phase after SE promotes the proliferation of neural stem cells and can prevent the loss of neurons in the CA1 and CA3 regions of the hippocampus (Han et al. 2021).

Interestingly, blocking the VEGFR-2 signaling pathway can inhibit vascular remodeling in the latent phase (Han et al. 2021) and prevent the disassembly of TJ proteins after kainate-induced seizures (Morin-Brureau et al. 2011). VEGF is overexpressed in neurons and glia in several brain regions including the hippocampus, thalamus, amygdala, and neocortex 24 hours after SE. In addition, exogenous VEGF protects hippocampal neurons following SE (Nicoletti et al. 2008). VEGF treatment during SE significantly prevented hippocampal astrogliosis (Lenzer-Fanara et al. 2017) and protected anxiety functioning, learning, and memory after SE (Nicoletti et al. 2010). The administration of high doses of VEGF directly into the cerebral tissue induces inflammatory and angiogenic responses that could promote BBB dysfunction. However, low doses do not induce inflammation or angiogenesis, but exert neuroprotection (Ruiz de Almodovar et al. 2009), highlighting the dual effects of VEGF in a dose-dependent manner. In addition to its protective effects on neurons and endothelial cells, VEGF has also been implicated in BBB breakdown after seizures and in mediating inflammatory responses (Rigau et al. 2007; Ruiz de Almodovar et al. 2009; Morin-Brureau et al. 2011; Ureña-Guerrero et al. 2020). VEGF causes inflammation characterized by principally monocytic infiltration initiated by a chemoattractant effect and upregulation of inflammatory cytokines. In addition, VEGF is upregulated by inflammatory mediators (Croll et al. 2004). Specifically, seizures induce overexpression and activation of VEGF/VEGFR-2, which, in turn, promotes angiogenesis and vascular permeability by the activation of collagenase, heparinase, plasminogen activators, and MMPs. VEGF overexpression was observed in neurons and astrocytes, whereas VEGFR-2 overexpression was found in capillaries and neurons (Morin-Brureau et al. 2012). VEGF-A promotes BBB breakdown by downregulation of claudin-1, claudin-5, and occludin. Angiogenesis is associated with increased BBB permeability through VEGF-induced inflammation, affecting the local vascular network, which triggers neuroinflammatory factors and promotes atrophy and seizure propagation (Baruah et al. 2020; Ureña-Guerrero et al. 2020). The phosphorylation of VEGFR-2 on Y1054/Y1056, which is necessary for maximal activation, was increased at 2 hours post-SE, parallel with the VEGF-induced Src activation and ZO-1 downregulation. Src inhibition provided effective protection of the ZO-1 network after SE, showing the main role of Src in ZO-1 downregulation (Morin-Brureau et al. 2011). In addition, in human pharmacoresistant epilepsies, VEGF-A and its receptor VEGFR-2 are increased (Fig. 13.2) (Rigau et al. 2007; Morin-Brureau et al. 2011;

Sun et al. 2016; Castañeda-Cabral et al. 2019, 2020b). Recently, it was shown that VEGF-A and VEGFR-2 are increased in the microvessels of patients with hippocampal sclerosis-TLE and lesion-TLE (Castañeda-Cabral et al. 2020b), which indicates that VEGF-A/VEGFR-2 are increased after seizures independently of the etiology of epilepsy.

In addition to its role in angiogenesis, VEGF also regulates lymphangiogenesis by VEGF-C/VEGFR-3. However, the signaling pathway in lymphatic endothelial cells upon VEGF-C binding to VEGFR-3 is less well characterized (Ruiz de Almodovar et al. 2009; Secker and Harvey 2021). The binding of VEGF-C to its receptor VEGFR-3 induces receptor dimerization, autophosphorylation, and activation of signaling pathways including ERK1/2 and Akt, which regulate the lymphatic endothelial cell survival, proliferation, and migration (Secker and Harvey 2021). Although it has been shown that the localization of VEGF-C/VEGFR-3 expression is widely distributed in the CNS, its role in neurological diseases is less well understood. Recently, Cho and colleagues showed that VEGF-C and VEGFR-3 are significantly overexpressed, mainly in the hippocampal astrocytes and microglial cells after SE. These increases in the VEGF-C and VEGFR-3 after acute seizures may promote neuroprotection (Cho et al. 2019). Furthermore, VEGFR-3 and mTOR are upregulated in reactive astrocytes after pilocarpine-induced SE. Interestingly, suppressing VEGFR-3 by selective inhibitor SAR131675 reduced glutamate transporter-1 expression and astroglial activation after SE, suggesting that VEGF-C/VEGFR-3 may be related to hippocampal astrogliosis after SE. Therefore, modulation of VEGFR-3 expression could prevent epileptic progression by restricting the aberrant cellular and network activity (Jeong et al. 2021). On the other hand, in human epileptic tissue, elevated expression of VEGF-C and VEGFR-3 was observed (Sun et al. 2016; Castañeda-Cabral et al. 2019). This evidence highlights the potential role of the VEGF-C/VEGFR-3 signaling pathway in epilepsy (Fig. 13.2).

13.6 Conclusions

The permeability of the BBB is a determining factor in the progression of different neurological disorders such as DRE and is also involved in the outcome and therapeutic efficacy of pharmacological treatments. Seizures induce damage to BBB and trigger an inflammatory response that promotes pathological neurovascular remodeling, aberrant angiogenesis, and neuronal dysfunction (Marchi and Lerner-Natoli, 2013; van Lanen et al. 2021; Tregub et al. 2022). BBB leakage is related to the occurrence of seizures and suggests that BBB dysfunction can further contribute to epileptogenesis, progression, and establishment of pharmacoresistant epilepsy (van Vliet et al. 2015; van Lanen et al. 2021). Antiangiogenic therapies could reduce the epileptogenic process by modulating BBB dysfunction mainly through modulation of VEGF signaling (Morin-Brureau et al. 2012; van Lanen et al. 2021). Therefore, evaluation of the BBB after seizures is relevant in determining the

progression of the disorder to epileptogenesis and pharmacoresistance, and developing strategies to avoid or delay those outcomes. On the other hand, regulation of the VEGF signaling pathways after seizures is also relevant for avoiding increased BBB permeability and preventing epileptogenesis and pharmacoresistance.

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Chapter 14

The Role of JNK3 in Epilepsy and Neurodegeneration



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Abstract N-terminal kinases (JNKs) belong to the subfamily of mitogen-activated protein kinases (MAPKs). JNKs are considered central signal transducers in the mammalian brain that are involved in many cellular processes, like regulation of gene expression, proliferation, and programmed cell death. Several in vitro and in vivo studies have reported alterations of the JNK pathway potentially associated with neuronal death in epilepsy and other neurodegenerative disorders. Ten JNK isoforms resulting from alternative splicing of three genes (*Jnk1*, *Jnk2*, and *Jnk3*) have been identified. They exhibit differences in tissue distribution and cell location. Despite the difficulty of assigning a specific JNK isoform function, JNK3, mainly located in brain, is responsible for several hallmarks associated with epilepsy and other neurodegenerative disorders, including apoptosis, oxidative stress, synaptic dysfunction, spine loss, excitotoxicity, mitochondrial dysfunction, and neuroinflammation. In this chapter, there is a description of the role of JNK3 in epilepsy and other neurodegenerative diseases. Highlighting that in epilepsy, the different JNK isoforms would be related to disease progression and that JNK activity modulation would be a good target to ameliorate the epilepsy pharmacoresistance. Likewise, there is a relation between the different compounds created against JNKs, underlining the difficulty of finding specific isoform JNK

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drugs. Selective isoform inhibition would have more efficacy than a general JNK activity inhibition.

Keywords c-Jun N-terminal kinase · JNK3 · Epilepsy · Neurodegeneration · JNK inhibitors

14.1 Introduction

One of the biggest health problems that we have today is the development of effective drugs for the treatment of neurodegenerative diseases. The cause is clear, the population over 65 years old is growing, at least in developed countries, and this implies the emergence of neurological diseases. Thus, in the next coming years, one of the main objectives in biomedical research will be to identify new targets for developing drugs that will stop or slow down the advancement of neurodegenerative diseases.

Distinct cell processes can participate in neuronal loss induction, such as alterations in cell cycle, oxidative stress increase, cytokine activation, and endoplasmic reticulum dysfunction, among others. There are also specific cell pathways, like Akt-GSK3 β , JAK1/STAT1, and MAPK mitogen-activated protein kinases (MAPKs), which are involved in triggering neuronal degeneration. In this way, the c-Jun N-terminal kinases (JNKs), a subfamily of MAPKs, are considered as central signal transducers in the mammalian brain, mostly implicated in mediating the apoptotic response of neurons (Bevilaqua et al. 2003; Levy et al. 2009). Therefore, JNKs have been considered as interesting targets to develop new drugs for the treatment of neurodegenerative disorders (Borsello and Forloni 2007; Braithwaite et al. 2010).

In multicellular organisms, in addition to the JNK pathway, three classical subfamilies of MAPKs have been described. Thus, there are the extracellular signal-regulated kinase 1 and 2 (ERK1/2) which mainly regulates cell growth and differentiation; p38 cascade is subdivided into two subgroups, p38 α /p38 β and p38 γ /

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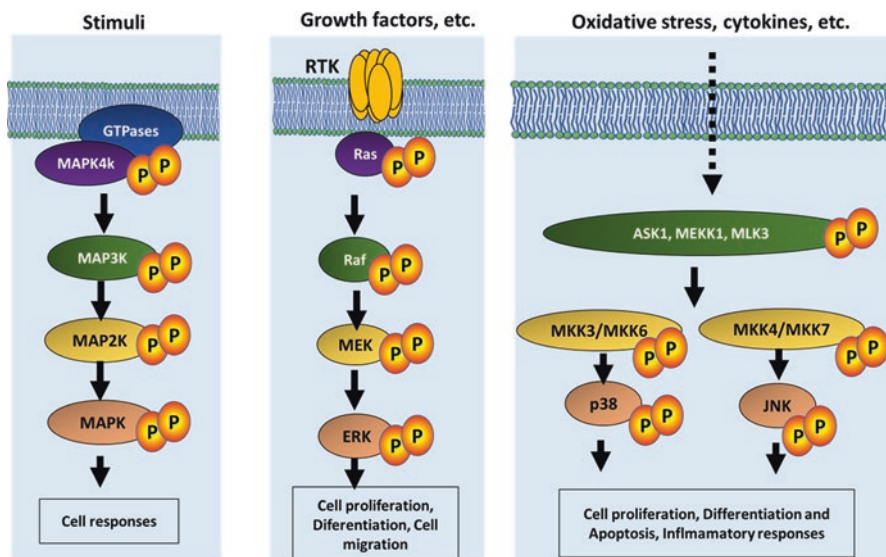


Fig. 14.1 MAPK proteins activate different cell pathways

p38 δ that together with (JNK) 1/2/3 signaling pathway have an important role in stress response like apoptosis and inflammation. Lastly, ERK5 regulates processes such as cell proliferation and differentiation (Fig. 14.1.). Furthermore, exist other atypical MAPKs, like ERK3/4, ERK7/8, and NEMO-like-kinase (NLK) that have unique regulation and function. All these MAPKs allow the cell to respond to exogenous and endogenous stimuli, integrating signals into cytoplasmic complexes and regulating the gene expression (Bozyczko-Coyne et al. 2002; Brecht et al. 2005; Borsello and Forloni 2007; Chen et al. 2019).

14.2 JNK Pathway Signaling

The JNKs activation is done through dual phosphorylation on Thr and Tyr residues by mitogen-activated protein kinase kinase 4 (MKK4) and kinase kinase 7 (MKK7) that, in turn, are phosphorylated by MAPK kinase kinase (MAP3K) (Fig. 14.1) (Weston and Davis 2007).

Scaffold JNK-interacting proteins (JIP) and β -arrestin 2 (β -Arr2) facilitate this sequential JNK phosphorylation cascade. β -Arr2 also acts as a scaffold protein for the ERK signaling pathway. The inactivation pathway is mediated by MAPK phosphatases (MKPs) (Fig. 14.2).

The activated JNKs, following a range of different stimuli, can phosphorylate and modulate the activities of hundreds of cytosolic and nuclear substrates (Borsello and Forloni 2007). Among the nuclear substrates identified, there are some hormone receptors, and transcription factors, such as the activator protein-1 (AP-1), the

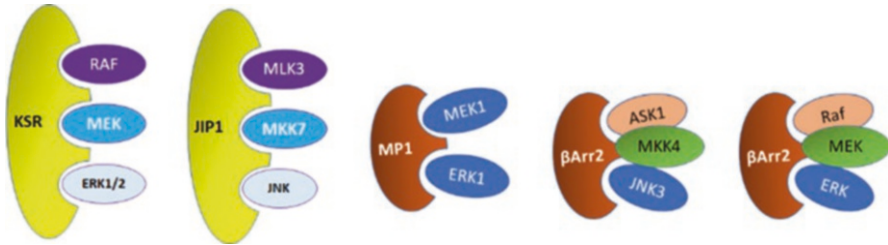


Fig. 14.2 MAPKS are activated by interactions between different scaffold proteins. Kinase suppressor of Ras: KSR; JNK-interacting protein1: JIP1; MEK partner1: MP1; β -Arr2: β -arrestin 2

family of Jun factors (c-Jun, JunB, JunD), Elk-1, p53, ATF-2, JDP2, c-Myc, the NAFT family, the STAT family, the PAX family among others. Their phosphorylation can mediate actions via a direct link to changes in gene expression. The activated JNK substrates in cytosol and other cellular compartments provide a link to a wide range of cellular functions, including cell death and cell movement, as well as allowing for modulation of other signaling events in the cell (Bogoyevitch and Kobe 2006). The high number of JNK substrates explains why this pathway, largely linked to cell death and inflammation processes, has been related to neuronal proliferation, differentiation, migration, and synaptic plasticity (Sun et al. 2015; Benoit et al. 2021).

Currently, the most studied substrate is the transcription factor c-Jun. Interestingly, the high levels of c-Jun, precede or coincide with periods of cell death, and they occur during embryonic development (Herdegen et al. 1997; Coffey et al. 2000) after brain trauma (Bozyczko-Coyne et al. 2002; Suckfuell et al. 2007), in cerebral ischemia (Tian et al. 2005), and following seizures (Gass et al. 1993; de Lemos et al. 2010). This link between the levels of JNKs and neuronal death supports the role of the JNK signaling pathway in epilepsy and several other neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and cerebral ischemia (Resnick and Fennell 2004; Ahmed et al. 2020).

In humans, three distinct genes (*Jnk1*, *Jnk2*, and *Jnk3*) each one located on a different chromosome, give rise to at least 10 different JNK isoforms that display differences among them, suggesting different functions, despite redundancy. Specifically, four splice forms arise from the *Jnk1* gene, four arise from the *Jnk2* gene, and two arise from the *Jnk3* gene seizures (Bogoyevitch et al. 2004). JNK1 and JNK2 are widely distributed in all organism tissues, while JNK3 is mainly located in the brain. In rodents, the *Jnk3* mRNAs are more highly distributed in the brain, where *Jnk3* mRNAs are highly distributed throughout all areas, whereas *Jnk1* and *Jnk2* mRNAs are mainly located in the cortex and the hippocampus, supporting the crucial role of JNK3 in neurodegenerative disorders despite their control in other cell functions such as differentiation cell proliferation, apoptosis between others (Carboni et al. 1998; Lee et al. 1999) (see Fig. 14.1). Likewise, JNK1 is involved in neuronal death (de Lemos et al. 2018), although it also has a crucial function in development and in the control of brain plasticity. This is supported by

the high levels of JNK1 in CNS during embryonic period followed by a noticed decrease during postnatal stages, together with the elevated amounts maintained in adult olfactory bulb (Carboni et al. 1998; Castro-Torres et al. 2020a, b). In this way, mice null for the *Jnk1* gene have the anterior commissure disrupted and exhibit alterations in the organization of cortical area and, at 8-month-old, showed axonal and dendritic processes degeneration together with learning impairment and motor alterations. These alterations and dysfunctions evidence that JNK1 is essential for proper CNS development (Coffey 2014).

The importance of JNK1 and also JNK2 isoforms is observed with the double knockout mice *Jnk1*^{-/-}*Jnk2*^{-/-}, which die between embryonic day E11 and 12 because the animals fail to close the neural tube due to a decrease in apoptosis rate in the hindbrain neuroepithelium together with an apoptosis increase in the forebrain (Sabapathy et al. 1999). The partial expression of *Jnk2* (*Jnk1*^{-/-}*Jnk2*^{+/-} mice) allows the loss of tissue in the retina (retinal coloboma) together with severe development deficiencies that provoke their death after birth. While a partial expression of *Jnk2* is not enough to compensate for the lack of *Jnk1*, mono *Jnk* allelic expression of *Jnk1* in *Jnk1*^{+/-}*Jnk2*^{-/-} mice allows the animals to survive (Weston and Davis 2007). Other double JNK mutant mice such as *Jnk1*^{-/-}*Jnk3*^{-/-} and *Jnk2*^{-/-}*Jnk3*^{-/-} and single JNK mutant mice do not die.

All these results obtained with knockout mice suggest that JNK1 and JNK2, but not JNK3, have a redundant role in regulating apoptosis in specific brain areas during brain embryogenesis. In addition, indicate that JNK1 and JNK2 are necessary for cell death during neural tube formation and, in turn, for promoting cell survival during cerebral cortex development neuronal (Sabapathy et al. 1999).

In this chapter, we review the progress in understanding the role of JNKs, specifically JNK3, in the pathophysiology of epilepsy and neurodegenerative diseases, together with the use of potential JNK inhibitors to treat neurodegenerative disorders.

14.2.1 JNKs and Neuronal Death

Some mechanisms have been proposed to explain how the JNK pathway directs the whole process of neuronal death by apoptosis. In this way, JNKs have been related to regulate the apoptosis activity, controlling the levels of the pro- and anti-apoptotic proteins, members of the B-cell lymphoma 2 (Bcl-2) family (Sun et al. 2005; Björklom et al. 2008). The Bcl-2 family proteins can be divided into three major subgroups:

1. *Anti-apoptotic proteins*, such as Bcl-2, Bcl-XL, and Mcl-1, which typically share four conserved motifs, termed Bcl-2 homology (BH) domains that can form heterodimers with Bax, inhibiting mitochondrial cytochrome c release and protecting against cell death.

2. *The pro-apoptotic proteins*, such as Bax, Bak, and Bok, which typically have three BH domains but promote cytochrome c release and apoptosis.
3. *The BH3-only proteins*, including Dp5/HRK (death protein 5/harakiri), Bim (Bcl2-interacting mediator of cell death), Bid, Bad, Puma, and Noxa, which share the BH3 domain. Thus BH3-only proteins are critical initiators of apoptosis and are stringently regulated at the transcriptional and post-translational levels depending on the cell type and apoptotic stimulus (Morishima et al. 2001; Puthalakath and Strasser 2002) (Fig. 14.3).

Specifically, the JNKs phosphorylation of Bcl-2 and Bcl-XL proteins decreases their anti-apoptotic activity while the phosphorylation of the pro-apoptotic protein BAD at Ser-128 potentiates its pro-apoptotic effect. The pro-apoptotic proteins Bim and Bcl-2-modifying factor (Bmf) are also phosphorylated by JNKs, inducing their translocation to the mitochondria, where they promote the release of mitochondrial proteins such as cytochrome c, apoptosis-inducing factor (AIF), and other mitochondrial pro-apoptotic death mediators (Donovan et al. 2002; Bogoyevitch and Kobe 2006). Therefore, the Bcl-2 protein family plays an important role in regulating the release of cytochrome c and other pro-apoptotic proteins from the mitochondria, which in turn are regulated by JNK proteins. All these processes favor cell death induction through a caspase-dependent or independent pathway.

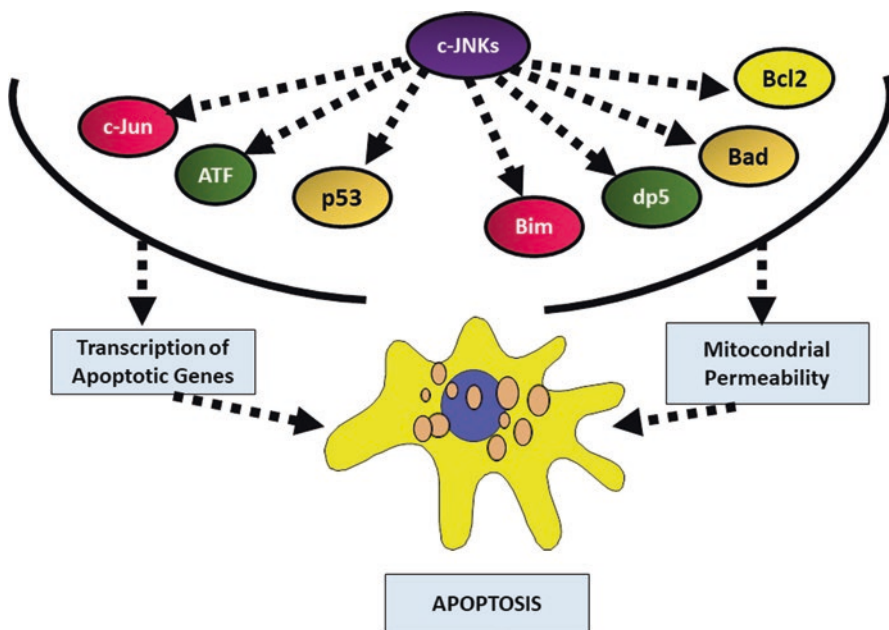


Fig. 14.3 Proposed mechanisms by which JNKs could regulate the apoptotic process. JNKs can induce the expression of nuclear genes that promote neuronal apoptosis. Furthermore, pro-apoptotic proteins can be phosphorylated by JNKs and then be translocated to the mitochondria to induce neuronal apoptosis

14.3 JNK Inhibitors

Since the activation of the JNK pathway may be a common step in neurodegeneration, the pharmacological inhibition of JNKs is a potential strategy to protect against neuronal loss. Although many JNK inhibitors have been discovered in the past decade, not all of them have reached clinical trials. This section highlighted some of the most important chemical inhibitors of JNK: CEP-1347, an inhibitor of the mixed lineage kinases MLK family responsible for the activation of MKK4 and MKK7, as well as SP600125, AS601245, CC-930, and AS602801 as selective inhibitors of JNKs activity (Lund et al. 2005; Carboni et al. 2004; Chen et al. 2010; Chambers et al. 2011). Looking for JNK isoform-specific inhibitors, Zheng et al. developed an aminopyrazole derivative compound with more than 30-fold selectivity against JNK3 than against JNK1 and JNK2 (Zheng et al. 2014). Later, Dou et al. reported that indolin-2-one derivative (J30-8) and quinoxaline derivative (J46) exhibited highly potent JNK3 inhibitory ability over 2500-fold and 400-fold isoform selectivity against JNK1/2 respectively (Dou et al. 2019; Dou et al. 2020). FMU200 and Rasagline are chemical compounds identified as more specific inhibitors of JNK3 (Yang and Gao 2018; Rehfeldt et al. 2021).

14.3.1 Characterization of JNK Inhibitors

CEP-1347 compound is a potent inhibitor of the mixed lineage MLKs that attenuates the activity of the downstream proteins JNK/c-Jun in neurons exposed to stressor factors (Saporito et al. 2002). Importantly, it is orally bioavailable and crosses the blood-brain barrier. CEP-1347 has shown neuroprotective effects both in vitro and in vivo against β -amyloid toxicity, trophic withdrawal in PC12 cells, MPP⁺ exposure, and apoptosis in cerebellar granule cells following serum and potassium deprivation (Maroney et al. 1998). Indeed, it has been observed that it reduces cytokine production in murine and human microglia cultures and inhibits TNF brain production in mice treated with lipopolysaccharide (Lund et al. 2005). Moreover, besides inhibiting the pro-apoptotic JNK pathway, this drug activates the levels of neurotrophins, such as Brain-Derived Neurotrophic factor (BDNF), as was observed in a mouse Huntington's disease (HD) model. It has also been shown that CEP-1347 increases BDNF mRNA levels in the brain of an experimental model of HD (R6/2 mice) compared to the control, which correlates with a reduction of HD progression in R6/2 mice (Apostol et al. 2008). Moreover, the PRECEPT clinical trial showed that CEP-1347 was safe and well-tolerated in a randomized placebo-controlled study in PD subjects; however, this drug failed to show efficacy in treating PD (The Parkinson Study Group PRECEPT Investigators 2007). A plausible explanation of the limited effect of CEP-1347 in neurodegenerative disorders is its poor selectivity since it acts upstream of JNK activators, the MLKs, blocking the JNK pathway in all neural cells (Saporito et al. 2002).

Drug repositioning studies have proposed CEP-1347 as a potential candidate for cancer stem cell-targeted therapy. For this reason, more clinical and preclinical studies are being carried out to evaluate its efficacy in cancer treatment (Okada et al. 2016; Singh et al. 2021).

SP600125 (anthra[1,9]-pyrazol-6(2H)-one) acts as a reversible ATP-competitive inhibitor of JNKs.

Costello and Herron showed that beta-amyloid peptide (A β -amyloid) fragment 25–35 impair posttetanic potentiation (PTP) and long-term potentiation (LTP) in the CA1 area on in vitro rat hippocampal slices, highlighting that JNK pathway interferes in the control of synaptic activity (Costello and Herron 2004). Interestingly, SP600125 restored these effects, increasing synaptic transmission, and enhancing PTP, both with or without the presence of A β . The data support the role of endogenous JNK activity in neurotransmitter control (Costello and Herron 2004). Likewise, in vivo studies using SP600125 in A β - injected rats induced potent memory-enhancing effects and blocked learning deficits, evidencing the neuroprotective capacity of SP600125 against A β (Ramin et al. 2011). Bennett and coworkers also demonstrated the neuroprotective effects of SP600125, since the compound inhibited the phosphorylation of c-Jun in Human CD4⁺ cells activated with anti-CD3 and anti-CD28. Besides, the expression of several inflammatory genes (*interleukin-2*, *IL-2*, *interferon-g*, *IFN- γ* , *tumor necrosis factor- α* *TNF- α* , and *cyclooxygenase-2*, *COX-2*) was prevented. Moreover, in vivo studies with CD-1 mice treated with lipopolysaccharide reported that SP600125 blocked the expression of *TNF- α* and inhibited anti-CD3-induced apoptosis of CD4⁺ CD8⁺ in thymocytes. All these results supported that targeting JNK would be an important strategy in inflammatory disease and apoptotic cell death (Bennett et al. 2001).

The neuroprotective capacity of SP600125 has been evidenced against different neurotoxins in in vivo studies. Thus, Chen and collaborators reported that the high levels of JNK phosphorylation observed in the hippocampus of amygdala-kindled rats' model, an experimental model of temporal lobe epilepsy (TLE), was inhibited by SP600125, evidencing their high specificity against JNK pathway and its probable effectivity in epilepsy treatment (Chen et al. 2010). This useful capacity in epilepsy was supported by Murphy's research group since the intracerebroventricular administration of SP600125 in mice previously injected with kainic acid (KA), a potent central nervous system excitant that is used in epilepsy research to induce seizures, blocked the induction of pro-apoptotic BH3-only protein Bim and CHOP. The data also reinforced that these proteins are activated by the JNKs (Murphy et al. 2010). Moreover, it has been observed that SP600125 exerts neuroprotective effects against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), considered a neurotoxin which causes permanent symptoms of Parkinson's disease, inhibiting JNK signaling and reducing COX-2 expression (Wang et al. 2005). In turn, Guan evidenced the neuroprotective effect of SP600125 in a rat model of transient global ischemia since an increase of surviving cells in the hippocampal CA1 subfield and a decrease in the activation of p-JNK1/2 and p-JNK3 were detected. Moreover, the increase of phosphorylated-c-Jun (Ser63/73) and

phosphorylated-Bcl-2 (Ser87) significantly diminished with SP600125 treatment, supporting that the compound acts against JNK (Guan et al. 2005).

An inhibitor with the ability to interfere with all JNK isoforms will be expected to be desirable for some therapeutic applications. However, we must stand out the disadvantages of SP600125 that limit its usefulness in human treatment, such compound as its poor stability in water. Moreover, since the JNK isoforms have different specific functions (Manieri and Sabio 2015; de Lemos et al. 2018), the discovery of new SP600125 derivatives with isoform selectivity its necessary. In this regard, the AJ-292-42151532 molecule has more specificity for JNK3 than the other isoforms, showing better efficacy (Rajan and Ramanathan 2020).

AS601245 is an orally, selective, ATP-competitive inhibitor that blocks the activity of the three human JNK isoforms, hJNK1, hJNK2, and hJNK3. Studies with this compound evidenced a decrease in TNF- α release and neuroprotective effects in in vivo models of ischemia (Carboni et al. 2004).

Bentamapinod (AS-602801) is another ATP-competitive JNK inhibitor that was used in clinical trials to treat inflammatory endometriosis; however, the results were not successful in CA3 areas (Palmer et al. 2016). Nevertheless, in vitro studies evidenced its cytotoxicity against nonstem cancer cells and cancer stem cells derived from human pancreatic, lung, ovarian, and glioblastoma cancer cells (Okada et al. 2016).

D-JNK-permeable peptide 1 or XG-102 As mentioned above, JNK activity can be regulated by JNK-interacting proteins, such as JIP-1, a protein that integrates the positive and negative regulators of JNK, facilitating the activity of the JNK signaling pathway. In mice, JIP-1 contains a JNK-binding domain (JBD) that mediates the sequestration of JNK in the cytoplasm, thus inhibiting the expression of genes that are activated via the JNK signaling pathway. Therefore, it acts as a functional JNK inhibitor. These properties of JIP proteins make them a good target to inhibit JNK activity. Therefore, JIP-derived peptides have been developed as the TAT-fused JNK-inhibiting peptide brimaptide (XG-102), also called D-JNK-permeable peptide 1 (D-JNKI). This compound has high cell permeability and, instead of inhibiting the enzymatic activity of JNKs, as classical chemical inhibitors do, blocks selectively the access of JNK to different substrates, hence preventing protein–protein interactions without interfering with its activation (Reinecke et al. 2012). XG-102 exerts neuroprotective effects against different in vitro models of excitotoxicity and has a neuroprotective role in in vivo experimental models, like cerebral ischemia, preventing cell death by apoptosis (Pan et al. 2010). Moreover, XG-102 has been observed to show beneficial effects on both hair cell death and the permanent loss of hearing induced by sound trauma (Wang et al. 2005). Finally, it could improve hemorrhage, retinal neovascularization, retinal excitotoxicity, and metabolic syndromes (diabetes, atherosclerosis) (Reinecke et al. 2012).

It is interesting to consider the otoprotective potential of another inhibitor of the JNK stress kinase, the *AM-111* (brimaptide; Auris Medical AG, Basel, Switzerland), a 31-amino acid cell-permeable peptide. A double-blind, randomized, placebo-controlled phase 3 study in patients suffering from severe-profound hearing loss and

treated with a single dose of AM-111 at 0.4 mg/ml showed a clinically relevant and nominally significant improvement in hearing and speech discrimination as well as more frequent tinnitus remission compared with placebo. Furthermore, the study confirmed that cochlear injury is associated with the activation of the JNK stress kinase pathway (Staecker et al. 2019).

Tanzisertib (CC-930) [(1S,4R)-4-(9-(S) tetrahydrofuran-3-yl)-8-(2,4,6-trifluorophenylamino)-9H-purin-2-ylamino) cyclohexanol] is a potent and orally active selective inhibitor of JNK1/2/3 isoforms with IC50s of 61 nM, 7 nM or 6 nM, respectively, and furthermore, it acts and against MAP kinases ERK1 and p38 with IC50 of 0.48 and 3.4 μ M, respectively. Tanzisertib has been used in clinical trials to study its capacity to treat fibrosis, discoid lupus, pulmonary fibrosis, and interstitial lung disease, among others. However, the results were disappointing (Plantevin Krenitsky et al. 2012). Nagy et al. identified new JNK inhibitory compounds with increased bias for JNK1, the isoform that emerged as the most critical one in driving fibrotic disease in both hepatic and lung fibrosis in animal models. Structure–activity relationship (SAR) optimization on a series of 2,4-dialkylamino-pyrimidine-5-carboxamides allowed the identification of compounds possessing low nanomolar JNK inhibitory capacity, overall kinome selectivity, and the ability to inhibit c-Jun. A compound with a potential capacity for clinical development was CC-90001. It was tested in clinical trials in patients with idiopathic pulmonary fibrosis ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT03142191) Identifier: NCT03142191) (Nagy et al. 2021).

14.4 JNK3 and Neurodegenerative Diseases

JNK3 and neurodegenerative diseases JNK3 is the JNK isoform mainly expressed in brain and most responsive to diverse hallmarks associated with neurodegenerative disorders, including apoptosis, oxidative stress, synaptic dysfunction, spine loss, excitotoxicity, mitochondrial dysfunction, and neuroinflammation. Specifically, it has been reported its role in the activation of the amyloid precursor protein (APP) promoting its conversion to A β -amyloid that trigger neuronal apoptosis in Alzheimer's disease (Yoon et al. 2012) as well as the mediation of neurotoxicity in rodent models of Parkinson's disease (Chambers et al. 2011). The selective expression of JNK3 in the brain, along with findings that Jnk3 knockout mice show neuroprotection against different neurotoxins, makes the inhibition of this isoform a promising therapeutic strategy to treat neurodegenerative diseases (Zheng et al. 2014; de Lemos et al. 2018; Rehfeldt et al. 2021). However, many already published specific JNK3 inhibitors are affected as well too, JNK1, JNK2, and p38 α too, because the three isoforms have a high amino acid sequence similarity in the ATP pocket that might induce potential side effects on immune and inflammatory systems (Colombo et al. 2009). Besides, the failure of pan-JNK inhibitors in clinical trials has brought attention to the development of JNK selective high inhibitors.

Dou et al. reported that the indolin-2-one derivative (*J30*–8) and quinoxaline derivative (*J46*) have high inhibitory ability against JNK3. They exhibit

neuroprotective activity *in vitro*, being a useful compound for the treatment of neurodegenerative diseases (Dou et al. 2020). Concretely, J30–8 can reverse Alzheimer's disease phenotype in APPswe/PS1dE9 double-transgenic mice by reducing the accumulation of A β and phosphorylation of Tau protein. *FMU200* is a chemical compound with known JNK3 inhibitory activity in *in vitro* cells. It decreased the neurotoxicity induced by 6-hydroxydopamine (6-OHDA) or hydrogen peroxide (H₂O₂) in undifferentiated SH-SY5Y cells and diminished cell death and cytokine production in LPS-treated RAW264.7 cells. This neuroprotection may result from suppressing the JNK pathways (Rehfeldt et al. 2021).

JNK3 activity is organized at spatial and temporal levels by its scaffold proteins, mainly JIP-1 and β -arrestin-2. Accordingly, in order to find specific chemical compounds against JNK3, to interfere the activity of their scaffold proteins, would be another way to block JNK3 function. Since JNK3 is detectable at the peripheral level, it could be used as a disease's biomarker. However, the capacity to obtain an early diagnosis of neurodegenerative diseases is still in the preliminary phase (Musi et al. 2020).

14.4.1 Epilepsy

Epilepsy is a heterogeneous group of chronic neurological disorders in which brain activity is abnormal; it is commonly associated with recurrent altered electrical brain activity, and seizures. Epilepsy, in addition to being an electrical disorder, can be considered a neurodegenerative disease because the vascular system plays an important role in inducing neuron loss and other features that occur in neurodegeneration disorders. The current therapy for epilepsy is based on the use of antiepileptic drugs (AEDs); however, 30–40% of patients do not respond to these treatments due to pharmacoresistance. Therefore, it is necessary to identify the cellular and molecular mechanisms responsible for medically intractable epilepsy to develop new targets.

JNK cell signaling pathway is a promising target in epilepsy control because it is strongly activated in the acute and chronic phases of epilepsy models (Zhang et al. 2018). In this way, Parikh et al. have shown that JNK hyperactivation is observed in animals with chronic epilepsy (at 6–9-week poststatus epilepticus), when animals have frequent convulsive seizures. Their results pointed out that JNK activation is not responsible for the process of epileptogenesis itself but may be a consequence of frequent convulsive seizures (Parikh et al. 2020). Furthermore, the importance of JNK activation in chronic epilepsy was also inferred using an array-comparative genomic hybridization that revealed that the JNK cascade plays a crucial role in congenital diseases associated with epilepsy, such as focal cortical dysplasia, polymicrogyria, and lissencephaly (Zhang et al. 2018). Moreover, posttraumatic epilepsy models treated with KA revealed astrocytic reactivity and increased JNK phosphorylation (Small et al. 2022). In addition, JNK1 gene expression is elevated in the temporal cortex of patients with temporal lobe epilepsy (TLE) (Castro-Torres

et al. 2020b). The role of the JNK pathway in epilepsy was also evidenced by the inhibition of the JNK cascade, since using *Jnk* knockout mice, the epilepsy was reversed as occurred with JNK inhibitors, however in this case, with only 50% of inhibition (Tai et al. 2017; de Lemos et al. 2018).

***Jnk* Knockout Mice Have Neuroprotection Against Seizure Induction**

As it was mentioned above, the single *Jnk* gene mammalian knockouts are viable, and they have enabled to elucidate the role of JNKs isoforms in a model of excitotoxicity as kainic acid (KA) that mimics the model of TLE in humans, inducing recurrent seizures and epileptiform activity in rodents. The administration of KA triggers a massive release of glutamate, which activates glutamate receptors leading to an increase in intracellular Ca^{2+} that in turn activates intracellular signaling cascades within susceptible neurons resulting in neuronal death.

One of these signaling cascades is the JNK pathway and in particular the increase in JNK1 levels that began an accurate predictor of cell excitotoxicity, even more than the c-Jun levels (Schauwecker 2000). The importance of JNK1 in epilepsy was supported by the neuroprotection observed in *Jnk1* knockout mice treated with KA (de Lemos et al. 2018). Behrens A, et al., observed that the blocking access of JNKs to c-Jun may offer a suitable target in neuroprotection thus they reported c-Jun activation as cell death prediction. Accordingly, mice with an inactive form of the *c-jun* (Jun AA: alanine instead of serine at positions 63 and 73) showed resistance to excitotoxic neuronal death (Behrens et al. 1999). In addition, giving more support to JNK pathway in epilepsy, some studies demonstrated neuroprotection in *Jnk3*^{-/-} mice against KA. These animals were less sensitive to seizures and, consequently, to neuronal death in the hippocampal CA1 and CA3 areas (Yang et al. 1997; de Lemos et al. 2010; de Lemos et al. 2018).

Although *Jnk1*^{-/-} and *Jnk3*^{-/-} mice show neuroprotection against KA, this must be given in different ways because the pattern of gene apoptotic expression is different between *Jnk1*^{-/-} and *Jnk3*^{-/-} mice, both in physiological conditions and after KA injection. Accordingly, *Jnk3*^{-/-} mice treated with KA reduced the induction of *Mcl-1*, *Bcl-10*, and *Cradd* genes, which are related to the regulation of the extrinsic and intrinsic pathway of apoptosis, while the lack of *Jnk1* reduces the induction of *Cidea* and *Hells* genes, related to DNA regulatory mechanisms (de Lemos et al. 2018). Moreover, it was observed that *Jnk3*^{-/-} mice in physiological conditions increased p110-beta protein levels and PI3K activity due to an upregulation of the *pik3cb*. This gene selectively increases neuroprotective cell pathways (Junyent et al. 2011). On the other hand, *Jnk1* null mice did not show any changes in AKT activity in the hippocampus (Junyent et al. 2011).

The role of JNK1 and JNK3 in acute epilepsy contrast with the hyperactivation of JNK2 found in a model of chronic epilepsy, the pilocarpine-induced-status-epilepticus (Parikh et al. 2020). Consequently, it is necessary to analyze the differential role of JNK isoforms along epilepsy progression since the selective

isoform inhibition would have more efficacy than a general JNK activity inhibition and moreover, would minimize adverse cognitive effects in antiepileptic therapy.

Therapeutic Epileptic Treatments Are Correlated with JNK Activity Modulation

Currently, one of the approaches for treating epilepsy in different experimental models is to use several drugs that have the capacity to modulate the activity of the JNK signaling pathway. In this way, the neuroprotective role of ketogenic diet in mouse treated with KA is associated with a diminution of the phosphorylated form of the three JNK isoforms, followed by a decrease of proenkephalin protein (PENK) that has proconvulsant properties (Noh et al. 2006). Uncaria rhynchophylla (Ur) is a medicinal plant belonging to the Rubiaceae family used in traditional Chinese medicine. Treatments with Ur in KA rats reduced epileptic seizures. This effect has been associated with regulating the innate immune system by a diminution in the levels of superoxide anions, JNK phosphorylation, and NF-kappaB activation (Hsieh et al. 2009). Another compound, the Acylated ghrelin that decreases the expression of phospho-JNK in hippocampal pyramidal neurons, reverted the status epilepticus induced in immature rats by the pentylenetetrazol-epileptic model (Zhang et al. 2013). On the other hand, the co-administration of muscimol and baclofen turn out in neuroprotection in a mice model of KA, inhibiting the increased assembly of the GluR6-PSD-95-MLK3 and also suppressing the activation of JNK signaling pathway members, as MLK3, MKK7, and JNK3 (Li et al. 2010). Moreover, licochalcone A (Lic-A), with high specificity against JNK1, has shown neuroprotective effects against KA treatments (Busquets et al. 2018).

All these data support that targeting the JNK pathway might be a promising therapeutic application for preventing and treating epilepsy. In this sense, the CC-90001 compound, a 2,4-dialkylamino-pyrimidine-5-carboxamides, has high selectivity for JNK1. The potency of CC-90001 to JNKs was tested in *Jnk1*^{-/-} and *Jnk2*^{-/-} murine embryonic. It was >10-fold more potent in cells expressing only JNK1 than only JNK2. Moreover, CC-90001 significantly inhibited liver fibrosis in an experimental rat model fed with choline-deficient amino acid (CDAA) as was observed in *Jnk1*^{-/-} mice in this model. The treated animals showed a 91% reduction in p-c-Jun levels compared to vehicle-treated controls (Nagy et al. 2021). The efficacy of CC-90001 in liver fibrosis model following oral administration made it as an appropriate compound for clinical development. In particular, CC-90001 was tested in Phase II clinical trials for pulmonary fibrosis with a completion date on December 24, 2021, but currently the clinical trial is inactive. (U.S. National Library of Medicine. <https://clinicaltrials.gov/ct2/show/NCT03142191>, page seen January 2023). Nevertheless, that compound is in phase I for the treatment of advanced solid tumors, alone or in combination with chemotherapy or nivolumab (U.S. National Library of Medicine. <https://clinicaltrials.gov/ct2/show/NCT0562541>; page seen January 2023).

The Transport Activity of ABCG2 Protein, That Is Modulated by JNK Activity, Is Related to Epileptic Pharmacoresistance

The administration to chronically epileptic rats of SP600125 (SP) compound, a selective inhibitor of the JNK/c-Jun signaling pathway, with a high specificity for JNK1, produces an antiepileptic effect without behavioral abnormalities, a similar response to the effects obtained by Lamotrigine (LTG), an antiepileptic drug clinically validated (Tai et al. 2017). With the use of SP in HCPT-resistant colon cancer cell subline SW1116/HCPT was revealed that the JNK inhibition has the capacity to reverse the transport activity of ABCG2 protein, an ABC transporter that mediates chemotherapy resistance in tumor cells (Zhu et al. 2012).

P-glycoprotein (P-gp) is present in the blood-brain barrier (BBB) and serves to pump out structurally unrelated compounds, likely serving as a method for the removal of toxins (and drugs). This pump efflux has been found to be overexpressed in blood vessel endothelial cells following temporal lobe resection for intractable epilepsy and in seizure foci. In animal experimental models, P-gp upregulation has been seen following seizure induction and status epilepticus. Accordingly, P-gp upregulation may be one of the reasons to explain the pharmacoresistance observed in some epileptic patients as occur in cancer treatment with chemotherapy. Supporting this hypothesis, some studies demonstrated that P-gp inhibitors have a potential role in the enhancement of drug uptake into the target brain region. Therefore, one way to treat epileptic pharmacoresistant patients would be to add P-gp inhibitors to the medication used to treat epilepsy diseases (Schinkel et al. 1994; Davis et al. 2014).

It is relevant to consider that there are different works that evidence a correlation between the P-gp modulation and JNK pathway activity. In this way, treated multidrug resistance (MDR) hepatoma human cell line (R-HepG2 cells) with Pheophorbide-a, a derivative of chlorophyll-a, in combination with a Photodynamic therapy, revealed an MDR suppression that was correlated with a downregulation of P-gp and an increase of JNK activation that mediates intrinsic apoptotic pathway (Tang et al. 2009). Li et al., by the treatment of mammal cells (Caco-2 and HepG2 cells) with deoxynivalenol (DON), one of the most abundant mycotoxins, supported the link between P-gp and JNK. Specifically, activated JNK enhances the phosphorylation of AKT, thus promoting the translocation of activated NF- κ B to the nucleus to activate P-gp expression (Li et al. 2018). In addition, the use of ultrasound (FUS) technique combined with microbubbles (MBs), a noninvasive and target-specific drug delivery method, supported this link between P-gp and JNK activity. It was observed that FUS-MBs increase drug permeability by temporary disruption of the blood-brain barrier (BBB), observing a downregulation of P-gp protein levels and an increase in JNK phosphorylation in the vascular region of the stimulated hemisphere (Choi et al. 2019). Therefore, the JNK pathway would be a good target for treating epileptic pharmacoresistance.

TLR4 and JNK Activity to Be Considered in Epilepsy Pharmacoresistance

Another point to be considered as an alternative to overcome the pharmacoresistance of the available AEDs is the development of drugs that target-specific inflammation pathways since inflammatory mediators, like interleukin (IL)-1b and Toll-like receptors (TLRs), together with other factors, have been identified to be linked to epilepsy progression, both in epileptic experimental animal models and in patients. Specifically, the TLR4 signaling pathway is activated in the hippocampus of KA-induced immature rats and in KA-induced epileptic juvenile rats (Chen et al. 2015; Wang et al. 2019). Wang et al. found a miRNA (miR-181b) that could inhibit autophagy and apoptosis, by inhibiting P38/JNK signaling pathway via targeting TLR4 (Wang et al. 2019). Moreover, Maroso et al. observed that the use of a TLR4 antagonist blocks epilepsy seizures and has the capacity to reduce acute and chronic seizure recurrence (Maroso et al. 2010). All this data sustains the important role of the JNK pathway in epilepsy diseases and supports the idea that the use of miRNAs that have a significant role in regulating inflammatory pathways involved in epilepsy could be a new approach to overcoming pharmacoresistance problems in epilepsy diseases (Srivastava et al. 2016).

14.4.2 Alzheimer's Disease

Alzheimer's disease (AD) is currently the leading global cause of dementia in the world. In the initial stages, AD is characterized by a mild loss of memory and then progresses to a severe decline of cognitive performance in the advanced stages (Zhu et al. 2001; Xu et al. 2009; Mondragón-Rodríguez et al. 2010). AD is distinguished by a series of histological markers that include neurofibrillary tangles, senile plaques, and a large loss of neurons (Castellani et al. 2009). The loss of neurons is associated with apoptosis, which is probably mediated by several inducers such as reactive oxygen species, A β -amyloid, mitochondrial dysfunction, and an inflammatory process that induces microglial activation (Su et al. 2008). On the other hand, a very important point in AD is the formation of A β -amyloid fragments that are derived from amyloid precursor protein (APP) after cleavage by beta and gamma-secretase, respectively. The C-terminal intracellular region (AICD) of APP plays an important functional role in regulating APP metabolism (Słomnicki and Leśniak 2008). AICD contains eight potential phosphorylation sites, but one of them, specifically T668, is phosphorylated by several kinases, including GSK3 β , JNK3, Cdc2, and Cdk5. Likewise, these kinases are associated with neurotoxicity and have been implicated in neurodegenerative diseases. Interestingly, JNK3 is specifically involved in the physiological regulation of AICD during neuronal differentiation, suggesting a role of JNK3 in plastic cellular processes, such as synaptogenesis (Taylor Kimberly et al. 2005).

Different signaling pathways are activated in the neuronal death process, and among them, the c-JNKs pathway plays a prominent role. Xu et al., with in vitro neuronal cultures and using an inhibitor of MLK3 (K252a), showed that the MLK3–MKK7–JNK3 pathway is involved in A β -induced toxicity and that MKK4 and MKK7 serve different functions in A β -mediated JNK3 activation (Xu et al. 2009). Supporting these results, Morishima et al., using in vitro cultures of primary cortical and hippocampal embryonic neurons, evidenced that A β induces the activation of c-Jun in a JNK-dependent manner with a special role of JNK3. The lack of JNK3 leads to neuroprotection because the number of neurons that undergo A β -inducing neuronal apoptosis is reduced. However, c-Jun phosphorylation was not completely inhibited, indicating that JNK1 or JNK2 may be involved in this phosphorylation. In addition, the authors found that the inhibition of Fas ligand and Fas function led to a decrease in A β -induced apoptosis, suggesting that JNK3–c-Jun–Fas ligand–Fas signaling cascade is involved in A β -mediated death (Morishima et al. 2001). That the activation of JNKs is essential for mediating the response of neurons to A β and that JNK signaling is required for in vivo plaque formation was supported by the group of Mazzitelli (Mazzitelli et al. 2011). Moreover, an in vivo study that induced JNK functional loss of neurons in mice carrying familial AD-linked mutant genes (*creER^{T2}*, *APP*, and *PS1* transgenes) reported the existence of a positive correlation between the levels of amyloid plaques and JNK signaling activity (Gourmaud et al. 2015). In the same study, the authors detected the existence of interaction between the levels of JNK3 and A β 42 in the cerebro-spinal-fluid (CSF) of patients with AD. Highlighting that there was a mutual relation rate between JNK3 levels and cognitive decline (Gourmaud et al. 2015). Intracerebroventricular administration of SP600125 in A β -injected rats supported the role of this JNK pathway in AD development because was observed a noticed decrease in the levels of caspase-3, TUNEL positive cells, and COX-2 together with an increase in Bcl-2/Bax ratio (Ramin et al. 2011). Furthermore, treatments in the H4-APPsw cell line (over-expressing the human APP gene and carrying the double Swedish mutation) with D-JNK1 produced a significant drop in β APPs levels, A β fragments, and A β circulating oligomers (Colombo et al. 2009). The chronic treatment with D-JNK1 in an experimental mice AD model (TgCRND8 mice) allowed the rescue of cognitive dysfunction, memory impairment, and LTP (Sclip et al. 2011). Thus, the use of D-JNK1 was useful to evidence the role of JNK in in vivo and in vivo AD models and pointed out that this compound was useful to treat AD features.

The work reported by Vogel et al., with primary hippocampal neuronal cultures, evidenced that JNK activity is responsible for another histological feature of AD, the induction of tau phosphorylation together with changes in its localization within neurons. In addition, with in vitro phosphorylation experiments, the authors elucidated that JNK3 exhibited high levels of autophosphorylation, suggesting the importance of this isoform toward tau (Vogel et al. 2009).

14.4.3 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease. The mechanisms involved in its pathogenesis include oxidative stress production, mitochondrial dysfunction, and protein aggregation, which promote the loss of dopaminergic neurons in the substantia nigra pars compacta (Levy et al. 2009). Currently, the main problem of PD and all neurodegenerative diseases is that therapy is focused on symptomatic relief. It is necessary to develop neuroprotective therapies that will slow disease progression. The investigation of cell death mechanisms common to several models of experimental PD may identify new drug targets for PD treatment.

It has been reported that mice exposed to MPTP (1-methy-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin that inhibits mitochondrial complex I and replicates most of the neuropathological hallmarks of PD in humans, such as the degeneration of dopaminergic neurons of the substantia nigra and an increase of COX-2 that was mediated by JNK. Therefore, COX-2 induction might represent an important step in the cascade of molecular events leading to neuronal loss in PD, even if its upregulation occurs in other pathological conditions, including AD (Hunot et al. 2004).

The use of *Jnk*-deficient mice showed that the JNK signaling pathway has a critical role in the animal model treated with MPTP to induce PD (Saporito et al. 2000; Peng and Andersen 2003; Hunot et al. 2004; Pan et al. 2009; Choi et al. 2010).

Considering the role of JNK signaling pathway in the development of PD, it was proposed to use the inhibitor *CEP-1347* [3,9-bis[(ethylthio)methyl]-(8R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H, 11H-2,7b,11a-triazadibenzo(a,g)cycloocta(cde)trinden-1-one] of mixed lineage kinases MLK3, MLK1 and MLK2. This upstream JNK pathway inhibition blocks the activation of downstream JNKs and can rescue neurons undergoing apoptosis. CEP-1347 is orally bioavailable and brain penetrant (Maroney et al. 1998; Bozyczko-Coyne et al. 2001). However, a clinical trial using CEP-1347 (Parkinson Research Examination of CEP-1347 trial, PRECEPT) to treat PD was terminated because it failed to produce significant improvements (Shoulson et al. 2007). *CEP-11004* [3,9-bis-[(isopropylthio)methyl]-(8R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cycloocta(cde)trinden-1-one] is another mixed lineage kinases MLK3, MLK1 and MLK2 inhibitor that prevents c-Jun NH(2)-terminal kinase (JNK) pathway activation. This compound has been used in the cell culture model of nerve growth factor (NGF)-deprived sympathetic neurons, where was evidenced an increase in the mRNA and protein levels of the TrkA receptor and, in addition, the PI3-kinase pathway was activated. These results suggested that the direct inhibition of the JNK pathway requires the indirect activation of the PI3-kinase pathway via Trk activation to induce neuronal survival and trophism (Wang et al. 2005).

As midbrain dopaminergic neurons express TrkB receptors, Wang and collaborators hypothesized that inhibition of MLK, the JNK pathway will not keep survival and that the addition of BDNF is required to activate the PI3-kinase pathway. The authors suggested that this was the reason why the CEP1-347 inhibitor failed to delay disease progression in PRECEPT (Shoulson et al. 2008). Furthermore, as we previously discussed, JNK1 and 2 are ubiquitously expressed in adult tissues and have important physiological functions; hence, the side effects associated with the inhibition of these enzymes limit the tolerable doses of JNK inhibitors. Accordingly, general inhibition of all JNK isoforms, such as that achieved by CEP-1347, may be of limited benefit to treating neurodegenerative diseases. On the other hand, JNK3 is neural-specific and does not exhibit high basal activity in the brain. Therefore, selective or specific inhibition of the JNK3 isoform, considered one of the main targets in apoptosis, could be more useful in reducing PD progression. In this line, studies carried out by Yang and Gao revealed that the levels of JNK3 in the brains of PD experimental models would be associated with PD disease progression due to the accumulation of axonal lesions and cell body damage. For this reason, Hepp Rehfeldt et al. considered that the specificity of FMU200 on JNK3 would be a better candidate for PD treatment (Rehfeldt et al. 2021). Moreover, *Rasagiline* which is a specific inhibitor for JNK3, has the ability to recover initial PD pathogenesis (Yang and Gao 2018). Another compound that could be useful is *Tat-JBD* which targets the formation of the JIP-1-JNKs complex, decreasing the c-Jun phosphorylation and, in turn, the death of dopaminergic neurons (Pan et al. 2010).

14.4.4 *Huntington's Disease*

Huntington's disease (HD) is a progressive neurodegenerative disorder caused by an autosomal dominant mutation in either of the two copies of the *huntingtin* gene. Specifically, mutant, disease-causing alleles contain a trinucleotide repeat expansion of variable length, which encodes polyglutamine tracts near the amino terminus of the HD protein, huntingtin (Jackson et al. 1998).

Systemic administration of mitochondrial toxin 3-nitropropionic acid (3-NPA) to experimental animals, such as nonhuman primates and rodents, produces similar symptoms to those observed in human patients with HD. The toxin irreversibly inhibits the succinate dehydrogenase (SDH) enzyme, the main constituent of the mitochondrial respiratory chain complex (MCC II) (Garcia et al. 2002). In vivo and in vitro treatments of rats and primary striatal cultures with 3-NPA induced the JNK signaling pathway activation associated with neuronal death (Perrin et al. 2009). This neuronal loss depends on the c-Jun transcription factor because the expression of dominant negative *c-Jun* protects striatal neurons from cell death induced by 3-NPA. Likewise, JNK activation appears to be a major factor in the apoptotic death of HN33 line cells induced by polyglutamine-expanded huntingtin (Liu 1998). Mutant huntingtin with 48 or 89 polyglutamine repeats enhances JNK activation and may trigger apoptosis, while normal huntingtin with 16 repeats fails to activate

the JNK pathway. Despite the role of JNKs in the neuronal apoptosis of HD, the lack of *Jnk3* in mice had no neuroprotective effect against 3-NPA, in contrast to what occurs with the intraperitoneal injection of KA (Junyent et al. 2012). This suggests that although the JNK pathway may be activated in HD, JNK3 could not be the main responsible for neuronal death as occurs in other neurodegenerative disorders; consequently, other proteins different from those involved in the JNK pathway may be involved in the neuronal loss.

14.4.5 Ischemia

In cerebral ischemia, there is an enhancement of neuronal excitation together with a decline of inhibition activity that leads to an imbalance between the excitation and the inhibition that aggravates the neuronal injury. The mitochondrial release of apoptogenic proteins plays a critical role in neuronal death after ischemia induction, and activated JNKs have an essential function in triggering this mitochondrial apoptosis-signaling pathway, mostly through Bax and Bim mitochondrial translocation (Gao et al. 2005). Pirianov et al. demonstrated that in *Jnk3*-deficient mice, decreased caspase-3 cleavage and Bim/PUMA protein levels, coupled with the upregulation of AKT/FOXO3a proteins, suggesting that the primary mode of JNK3 action is to promote apoptosis. Moreover, the authors demonstrated that after hypoxic-ischemic induction in mice, at postnatal day 9, JNK3 activity triggers the transcription of many death genes through the p-c-Jun, including the pro-apoptotic Bcl-2 family member, Bim, and the death receptors TNFR (p55) and CD95/Fas (Pirianov et al. 2007). Qi et al. supported the role of JNK3 in neuronal death during ischemia–reperfusion. They reported that ethanol has neuroprotective effects by inhibiting the excitatory JNK3 apoptotic pathway by preventing the association of Gluk2-PSD-95-MLK3 signaling module. Their results evidenced that ethanol activates the presynaptic GluK1-KA receptor and facilitates the release of inhibitory GABA neurotransmitter that activates postsynaptic GABA_A receptors, which in turn suppress the ischemic depolarization, decreasing the association of Gluk2-PSD-95-MLK3 proteins (Qi et al. 2010). Furthermore, other studies evidenced that JNK3 signaling is implicated in the mitochondrial release of cytochrome c, leading to caspase-3 activation, either via a Bim-dependent mechanism or through direct targeting of mitochondria (Morishima et al. 2001; Murphy et al. 2010). In a rat model of cerebral ischemia–reperfusion was observed that JNK3 activation was mediated by one subunit of KA receptors, the GluR6, also called GluK2. The brain injury increased the GluR6-PSD95-MLK3 signaling module that activated JNK3 and their downstream nuclear and nonnuclear substrates (Tian et al. 2005; Lv et al. 2012; Jin and Bo 2021). The postsynaptic density protein 95 (PSD-95) is a scaffold protein characterized by the presence of several protein-binding domains, including three N-terminal PDZ domains, a signal Src homology region 3 domain, and a C-terminal guanylate kinase-like domain.

Moreover, the PDZ domains bind to the C-terminus of the NMDA receptor NR2 and KA receptor GluR6 subunit, which is crucial for the tethering of NMDA receptors and KA receptors in the postsynaptic membrane (Hu et al. 2008; Han et al. 2008). Jin and Bo confirmed that *sevoflurane*, a highly fluorinated methyl isopropyl ether, inhibits the over-activation of NMDA receptors and reduces the assembly formation of GluR-PSD95-MLK3. This action negatively regulates the MLK3-MKK7-JNK3 signaling pathway, potential therapeutic strategy for cerebral ischemia–reperfusion injury (Jin and Bo 2021). Song et al. described data supporting the role of the MLK3-MKK7-JNK3 signaling module in an experimental model of ischemic stroke. They demonstrated that Heme oxygenase (HO-1) could inhibit the assembly of the MLK3-MKK7-JNK3 signaling module scaffolded by JIP1 by diminishing the JNK3 phosphorylation (Song et al. 2019). Previous studies done by Ge et al. in an ischemia/reperfusion (I/R) rat model evidenced that *metformin* (used as antidiabetic drug) could attenuate the deficits of hippocampal-related behaviors and the inhibition of neuronal apoptosis through the activation of Akt1, the reduction of caspase 3 and the phosphorylation of JNK3/c-Jun.

Therefore, clinical trials are needed to know the effects of this drug on ischemia disease (Ge et al. 2017).

Another drug that can ameliorate the effects of ischemic stroke is *Cilostazol* (CTL), used as an anti-platelet agent. It could impair behavior and locomotor activities via the PI3K-Akt1/JNK3/caspase-3 mechanism (Qi et al. 2016).

Plotnikov et al. reported that *11H-indeno[1,2-b] quinoxalin-11-one oxime (IQ-1S)*, a selective JNK3 inhibitor, showed neuroprotective effects in rat experimental models of reversible focal cerebral ischemia by attenuation of the phospho-c-Jun increase in the hippocampus (Plotnikov et al. 2020).

The structural insights of recent FDA-approved medications (*dabigatram*, *estazolam*, and *pitavastatin*) revealed a significant contribution of the hydrophobic regions and significant residues to active site regions of JNK3. In an in vivo rat model of focal cerebral ischemia, the drugs restored the antioxidant enzyme activity and reduced the levels of oxidative stress and neuronal degeneration, possibly by inhibiting JNK3. These drugs might be clinically used for ischemic stroke-associated brain degeneration and other neurodegenerative diseases related to oxidative stress and neuroinflammation (Zulfiqar et al. 2020).

14.5 Future Perspectives of Inhibiting the c-JNKs Pathway in the Treatment of Neurological Disorders

The development of neuroprotective drugs is undoubtedly an area of increasing relevance due to the high incidence and prevalence of neurological disorders and the lack of effective treatments, as the pharmacoresistance of patients with epilepsy.

There are many cellular and biochemical parameters, such as oxidative stress, mitochondrial alteration, cell cycle reentry, cytoskeletal alteration, and inhibition of pro-survival pathways, that might contribute to the development of neurodegenerative diseases. Different studies reported that JNKs are involved in these biochemical

processes; therefore, targeting the JNK pathway with effective inhibitors would provide neuroprotection. Many JNK inhibitors have been discovered, and some have been introduced into clinical trials. Unfortunately, one of the candidates, the CEP-1347, used in clinical trials for PD treatment, did not succeed. One possible cause could be a failure of the dose used. Therefore, it is necessary to conduct more clinical trials with JNK inhibitors. In this way, the CC-90001 compound, with high selectivity for JNK1, is currently used in clinical trials for solid tumor treatments and pulmonary fibrosis.

Since more than one pathway may be involved in neuronal death, a possible alternative would be to consider treatments with an antagonist of JNKs together with other drugs, such as antioxidant. However, JNK pathway inhibition has limitations due to its biological functions. For example, it has been reported that JNK inhibitors can rescue axotomised neurons, but prevent their regeneration. For that reason, inhibiting specific JNKs is a challenging task. The benefits of selective inhibition could avoid side effects and have better therapeutic results; however, this requires a better understanding of the JNK isoforms function. The efficacy of selected inhibition was reported by Zhao et al., who demonstrated that by inhibiting specifically the mitochondrial complex MKK-JNK3, the apoptosis was attenuated without affecting cellular functions (Zhao et al. 2012). Likewise, when designing drugs against JNK, it must be considered that the nuclear localization of JNKs is the main factor responsible for cell death, while the cytoplasmic localization is responsible for its physiological functions (Björkblom et al. 2008).

Recently it has been described that combining drugs used in specific therapies with JNK inhibitors are able either to improve therapeutical effects or additionally decrease drug resistance. That strategy was tried for cancer treatment in several tumors (Gao et al. 2005; Inoue et al. 2018; Li et al. 2020). Accordingly, this therapeutic design could be considered for the treatment of neurodegenerative diseases, since the combination of JNK inhibitors with other neuroprotective drugs could play a synergistic neuroprotective role. Moreover, given that JNK inhibitors also could act on multiple signaling pathways simultaneously with other drugs, this approach could achieve a new neuroprotective effect and open a new window for the treatment of neurodegenerative disease (Zhu et al. 2022).

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Chapter 15

Application of Proteomics in the Study of Molecular Markers in Epilepsy



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Abstract Proteomics has helped us to understand physiological functioning as well as pathological condition. Mesial temporal lobe epilepsy (MTLE), the most common type of focal epilepsy in humans, is frequently associated with hippocampal cell loss, cognitive deficit, and resistance to antiseizure medications (ASM). A major challenge in designing new treatments to control seizures or epileptogenesis is the sequence of events involved in the development of an epileptic focus. Neuroproteomics emerges as a powerful tool for the assessment of protein biomarkers in neurodegenerative diseases, including MTLE. In this chapter, we present the main techniques used in proteomics studies to determine differential expression of proteins in brain tissue or other biological samples from patients and experimental models of epilepsy. Data obtained with proteomics are very useful to understand the pathophysiology of MTLE and, in the future, may assist in finding target proteins for new therapies.

Keywords Proteomics · Temporal Lobe Epilepsy · Hippocampus · Epilepsy model · Biomarkers

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15.1 Introduction

Proteomics is a science or method developed to study the proteome. Proteome is a term used to refer to the amount of protein expressed in a cell or tissue of an organism. While the genome reflects the sum of genes, the proteome does not exhibit a fixed number of proteins. These vary according to the state of development, the tissue type, and the physiological conditions. A proteome, nevertheless, remains a direct product of a genome. The number of proteins in a proteome can exceed the number of genes as protein products expressed by alternative splicing or with different post-translational modifications are observed as separate molecules on a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Wilkins et al., 1995).

Proteomics allows us to find proteins changed by a cell, tissue, or organism's response to internal states, external stimulations, or developmental changes, and to profile any differential protein expression (Mus-Veteau, 2002; Wang et al., 2010). Proteomics not only measures the amount of a given protein but also whether there are any modifications of a protein as phosphorylation, ubiquitination, palmitoylation, oxidation, and other post-translational modifications (PTMs) (Alzate, 2010). Proteomics is a multidisciplinary method that is based on principles of biochemical, biophysical, and bioinformatics to allow the distinguishing of healthy and diseased cellular processes at the protein level. Aslam et al. (2017) highlighted some aspects of a target organism's proteome that can be assessed by proteomics, for example, protein identification, protein quantification, protein localization, post-translational modifications, functional proteomics, structural proteomics, and protein-protein interactions.

Currently, the proteomics technique has been applied in the investigation of biomarkers associated with disease (Liu et al., 2010). However, proteomics technology is not only applicable to study health or disease but also in agronomy researchers (i.e., corn, sugarcane, rice, wheat, etc.) or organisms with economic impact (i.e., *Rhizobium* spp., which set nitrogen to legumes, or *Xanthomonas* spp., that cause diseases in bean or citrus cultivation) (Di Ciero & Bellato, 2003).

Proteomics technology applied to understanding the nervous system is called "neuroproteomics" or "neuromics". The neuroproteomics enables to study proteome of brain fragments or single cell, in cultures or isolated, and this is important to determine the dynamics of subproteome under different conditions (i.e., oxidative stress, drugs, etc.). In a global analysis, complementary studies could contribute to the understanding of complex biological networks that include protein interactions, complexity of signal and metabolic pathways that can be applied to select potential targets for specific drug therapy, and to the development of diagnosis or prognosis for neurological disorders (Liu et al., 2010).

15.1.1 Techniques Used in Proteomics

In a broad view, the techniques applied to proteomic analysis can be classified as follows: 1 – *Low-throughput analysis*, which includes: (A) *Antibody-based methods*, ex. ELISA (enzyme-linked immunosorbent assay) and Western blot. These methods rely on the availability of antibodies targeted toward specific proteins or epitopes to identify proteins and quantify their expression levels. (B) *Gel-based proteomics*, the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE or 2DE), the first proteomics technique developed. This technique uses an electric current to separate proteins differing in their isoelectric point in a gel (first dimension) and mass (second dimension). Differential gel electrophoresis (DIGE) is a modified form of 2DE that uses different fluorescent dyes to allow the simultaneous comparison of two to three protein samples on the same gel. These gel-based methods are used to separate proteins before further analysis by, e.g., mass spectrometry (MS), as well as for relative expression profiling. (C) *Chromatography-based methods*: can be used to separate and purify proteins from complex biological mixtures such as cell lysates. For example, ion-exchange chromatography, size exclusion chromatography, and affinity chromatography. These methods can be used to purify and identify proteins of interest, as well as to prepare proteins for further analysis (Aslam et al., 2017).

2 – *High-throughput analysis*, including: (A) *Analytical, functional, and reverse phase microarrays*. Protein microarrays apply small amounts of lysed samples to a “chip” where antibodies can be immobilized and used to capture target proteins in a complex sample. This is termed an analytical protein microarray. Functional protein microarrays are used to characterize protein functions such as protein–RNA interactions and enzyme-substrate turnover. The protein arrays provide a “map” of the activated signaling network by the quantitative analysis of the phosphorylated, or activated, state of cell signaling proteins. Reverse-phase protein microarrays have been developed to generate a functional “map” of the cell signaling networks based directly on cellular analysis of tissue samples (Fig. 15.1). In a reverse-phase protein microarray, proteins from, e.g., healthy vs. diseased tissues or untreated vs. treated cells are bound to the chip, and the chip is then probed with antibodies against the target proteins (Aslam et al., 2017; Chandramouli & Qian, 2009).

(B) *Mass-spectrometry-based proteomics*. Gel-free methods for separating proteins would include isotope-coded affinity tag (ICAT), stable isotope labeling with amino acids in cell culture (SILAC) and isobaric tags for relative and absolute quantitation (iTRAQ). These techniques allow either quantitation or comparative/differential proteomics. Besides that, a less quantitative technique called multidimensional protein identification technology (MudPIT) can also be used. Other gel-free chromatographic techniques for protein separation include gas chromatography (GC) and liquid chromatography (LC).

The low-throughput gel-based proteomic analysis has four basic stages: extraction, purification, separation, and identification of proteins. The 2D-PAGE allows the separation of hundreds to thousands of proteins in a single experiment (Van den

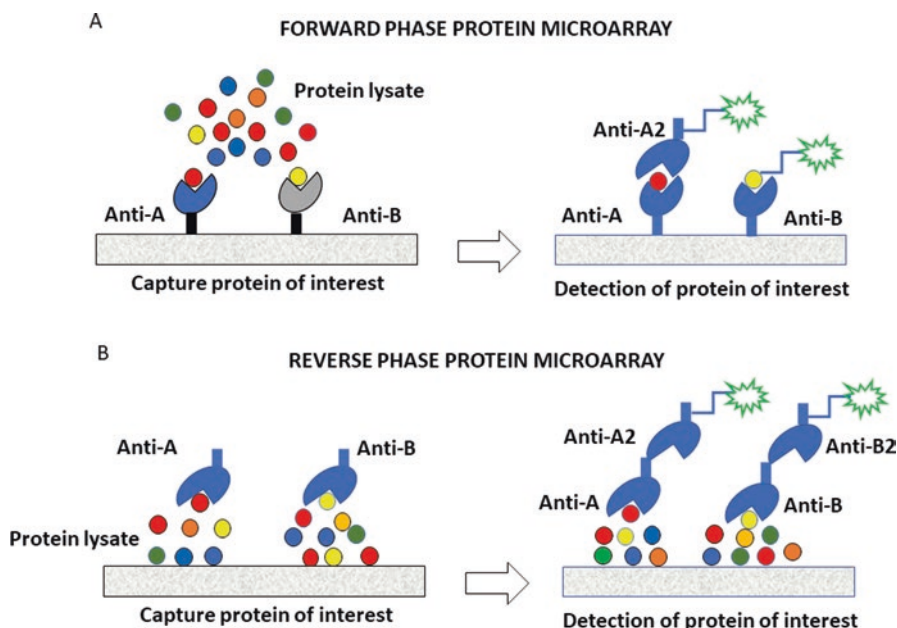


Fig. 15.1 Forward phase microarray (a) and reverse phase protein microarray (b). The forward phase microarray consists of the immobilization of antibodies on a solid surface of a chip, to capture proteins of interest, allowing direct comparison of different samples. In reverse-phase microarray proteins are immobilized on the chip surface, and the chip is probed with the antibodies. Reverse-phase array requires only one well-performing analyte detection reagent

Bergh & Arckens, 2005; Kim et al., 2007) and mass spectrometry (MS) to detect either digested peptides or intact proteins (Li & Smit, 2008; Zetterberg et al., 2008). MS requires peptides or proteins to be studied as ions in the gas phase matrix-assisted laser desorption/ionization (MALDI) and electro-spray ionization (ESI) are most used for this purpose.

There are five types of mass analyzers commonly used for proteomics research, and they vary in their physical principles and analytical performance (see Liu et al., 2010). MALDI-TOF (time-of-flight, TOF) is generally used in proteomics studies to identify the protein from in-gel digestion of gel-separated protein bands by peptide mass fingerprinting due to its excellent mass accuracy, resolution, and sensitivity (Pappin et al., 1993). Nowadays, it is possible to conduct a study of proteomics analysis without the need for two-dimensional electrophoresis. Different methods enable the analysis without 2-DE gels, one being accomplished by methodology 2D-LC-MS, in which the proteins are labeled with a probe, trypsinized, and then analyzed by LC-MS, which makes the simple and reproducible method, but it is more expensive.

Other protein identification methods, like amino acid composition analysis, N-terminal sequencing, or immunochemistry, as well as column chromatography, can be used (Fountoulakis, 2001), or other biochemical techniques can be applied for protein enrichment (Fountoulakis & Takács, 2002).

15.1.2 *Proteomics and Epilepsy*

Epilepsy is a chronic neurological disease that affect approximately 50 million people worldwide, being characterized by spontaneous and recurrent seizures that occur in the absence of disease toxic-metabolic or fever (Beghi, 2019). Temporal lobe epilepsy (TLE) accounts for approximately 40% of all cases of epilepsy, being a common form of focal epilepsy in humans. There are two subtypes of TLE, namely, mesial temporal lobe epilepsy (MTLE) and neocortical temporal lobe epilepsy (NTL) (Pfänder et al., 2002). In most MTLE patients, seizures originate in the limbic areas and are focal, and may be perceptual or nonperceptive (Tatum, 2012). Hippocampal sclerosis (HS) is the most frequent histopathological feature present in many patients with MTLE, and this change can usually be detected by magnetic resonance imaging – MRI (Lewis et al., 2014; Blumcke et al., 2017; Bruxel et al., 2021). HS is characterized by neuronal loss in specific subregions of the hippocampus and glial scar, but the etiology and the pathogenesis of the cell death are not well understood (Blumcke et al., 2017). In addition, synaptic rearrangement and cell scattering in the granular layer of the dentate gyrus are frequently seen associated with HS in MTLE (Houser, 1990).

Seizures are often frequent, and about 67–89% of patients with MTLE do not respond to antiseizure medications (Chipaux et al., 2016; Do Canto et al., 2021). According to the International League Against Epilepsy (ILAE), the drug resistance epilepsy (DRE) occurs as the failure of adequate trials of two tolerated, appropriately chosen, and used antiseizure medications, whether as monotherapy or in combination, to achieve sustained seizure freedom (Kwan et al., 2010). The concept of DRE not only implies intractable seizures, but the underlying pathogenesis is responsible for structural and neurobiochemical changes that also cause cognitive and neuropsychiatric disturbances and psychosocial dysfunction in patients with TLE (Kwan & Brodie, 2002; Fattorusso et al., 2021). Therefore, DRE represents a spectrum of different clinical and neurobiological pictures rather than a group of patients with the same disease, requiring a complex approach to several issues that we will further try to focus on.

Until now, there is no antiseizure medications able to prevent seizures in patients with TLE that are efficient in preventing epileptogenesis (Temkin, 2009). Thus, the question is whether the epileptogenic process could be explained by common molecular and network events that would be applied in new therapeutics. In this way, proteomics has been a powerful tool for protein profiling because it allows comparing proteomes of cells and tissues in normal and pathological conditions. Since the expression of proteins is determined, the transcriptional level can be examined to find the underlying mechanism for the reduction or increase of certain gene products. For this reason, proteomics has been widely used in clinical research to identify biomarkers associated with disease.

Neurosciences have benefited greatly from the increased use of the technique proteomics in recent years. Despite this, studies of epilepsies are still modest with the application of proteomics. Experimental models that reliably reproduce the

main symptoms of diseases have aroused the interest of researchers in the search for biomarkers. Proteomics has been used in epilepsy models and brain tissue or cerebrospinal fluid (CSF), obtained from patients with refractory seizures, to identify biological markers, enabling new treatments. In the next sections, we will briefly review these results.

Despite technological advances applied to neurosciences, little is known about the cellular and molecular phenomena related to the epileptogenic process (Silva & Cabral, 2008).

Proteomics Profile of Epilepsy Models

The use of experimental animal models of epilepsy has contributed greatly to the knowledge of different pathological mechanisms that are difficult to study directly in humans due to the limitations of human tissue availability (Becker, 2018). These models approach the reality of the complex and heterogeneous physiological processes involved, enabling the creation of therapeutic strategies for the disease. The most common acquired epilepsy model of MTLE in rats and mice are pilocarpine, kainic acid, and electrical stimulation. Some genetic models have also been largely studied due to the pathophysiological characteristics they present, and they are, Wistar Albino Glaxo/Rijswijk (WAG/Rij) rat, BS/Orl and BR/Orl mouse strains and Genetic Absence Epilepsy from Strasbourg (GAERS) (Becker, 2018). Each model produces different mechanisms that lead to epileptogenesis; therefore, not all have the same proteomic profile, although there are similarities in the immune response and inflammatory pathways (Pitkänen et al., 2015).

Although these findings are interesting and enable us to obtain clues about the mechanisms involved with intractable epilepsy, we must bear in mind that these clues refer to mechanisms already established and irreversible, such as cell loss, sprouting, cell dispersion, glial scar, metabolic changes, etc. (Pitkänen et al., 2015). Studies employing human tissue are limited by the low amount obtained by surgical procedures and for ethical reasons. The use of the experimental model of epilepsy can expand our knowledge regarding these mechanisms involved in epileptogenesis, allowing interference with or preventing the onset of spontaneous seizures.

Chemical convulsants such as pilocarpine (Cavalheiro et al., 1991; Cavalheiro, 1995) and kainic acid (Ben-Ari, 1985) can initiate *status epilepticus* in rodents and cause hippocampal sclerosis, memory impairment and spontaneous and recurrent seizures (Cavalheiro et al., 1994).

Using the proteomics method, we studied the differentially expressed proteins in the hippocampal samples of rats subjected to pilocarpine-induced MTLE. In the hippocampal samples from rats studied 90 days following status epilepticus, we found 40 proteins differentially expressed when compared to control animals. Among them, 37 proteins were successfully identified, as shown in Table 15.1. The protein profile revealed that 29 proteins were upregulated, 6 were downregulated and 2 were expressed only in control animals.

Table 15.1 Proteins differentially expressed in the hippocampi of rats subjected to pilocarpine

GeneCards	Protein name	Changes	IP	MW
Ldha	L-lactate dehydrogenase A chain	∅	5.5	36,874
Pebp1	Phosphatidylethanolamine-binding protein 1	∅	5.2	20,902
Aldoa	Fructose-bisphosphate aldolase A	↓	9.3	39,783
Pla2g4c	Cytosolic phospholipase A2 gamma (fragment)	↓	5.2	37,522
Abcb6	ATP-binding cassette subfamily B member 6, mitochondrial	↓	9.3	93,305,18
Mdh1	Malate dehydrogenase, cytoplasmic	↓	6	36,631
Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	↓	5.4	38,151
Gnb3	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	↓	5.3	38,125
Eno1	Alpha-enolase	↑	6	47,440
–	Enolase	↑	10.3	34,166
Eno3	Beta-enolase	↑	7.9	47,326
Eno2	Gamma-enolase	↑	4.8	47,510
Aldh5a1	Isoform Short of Succinate-semialdehyde dehydrogenase, mitochondrial	↑	9.4	53,391
Park7	Protein DJ-1	↑	6.2	20,190
Mapk1	Mitogen-activated protein kinase 1	↑	6.5	41,648
Tpi1	Triosephosphate isomerase	↑	7.9	27,345
Nsf	Vesicle-fusing ATPase	↑	6.5	83,170
Pgam1	Phosphoglycerate mutase 1	↑	6.6	28,928
Ywhag	14-3-3 protein gamma	↑	4.6	28,456
Ldhb	L-lactate dehydrogenase B chain	↑	5.5	36,874
RGD1565368	Glyceraldehyde-3-phosphate dehydrogenase-like	↑	9.3	36,045
Dpysl2	Dihydropyrimidinase-related protein 2	↑	5.8	62,638
Dpysl3	Isoform 1 of Dihydropyrimidinase-related protein 3	↑	5.9	62,327
Atp6v1b2	V-type proton ATPase subunit B, brain isoform	↑	5.4	56,857
Car2	Carbonic anhydrase 2	↑	6.9	29,267
Gfap	Isoform 1 of Glial fibrillary acidic protein	↑	5.1	49,984
Tubb5	Isoform 1 of Tubulin beta-5 chain	↑	4.6	50,095
Tubb2a	Tubulin beta-2A chain	↑	4.6	50,274
Tubb2c	Tubulin beta-2C chain	↑	4.6	50,225
Tubb3	Tubulin beta-3 chain	↑	4.6	50,842
Atp5b	ATP synthase subunit beta, mitochondrial	↑	4.9	56,318
Tpi1	Triosephosphate isomerase	↑	7.9	27,345
LOC500959	Triosephosphate isomerase	↑	6.4	27,306
Capzb	F-actin-capping protein subunit beta	↑	5.4	30,952
Wdr1	WD repeat-containing protein 1	↑	6.1	66,824
Atp6v1a	V-type proton ATPase catalytic subunit A	↑	5.2	68,564
Cdca71	Cell division cycle-associated 7-like protein (Cdca71)	↑	5.8	50,854

Proteins identified by LC-ESI-MS/MS and peptide matched by using Mascot MS/MS ion search and IPI protein database. (Filled up arrow) upregulated proteins; (open down arrow) downregulated proteins (pilocarpine versus control rats), and (∅) proteins expressed only in the hippocampus of control rats. MW: molecular weight; IP: isoelectric point

Among the downregulated proteins, we found enzymes related to carbohydrate metabolism and ATP synthesis, reflecting disturbances in the energetic metabolism. These data are in line with findings reported by other authors (Greenberg et al., 2005; Masino et al., 2009). The gene encoding the malate dehydrogenase was reported as a factor involved in the generation of generalized idiopathic epilepsy (Greenberg et al., 2005). Altered proteins such as phospholipase A2, fructose-bisphosphate aldolase, and enolase, have been reported by other authors associated with neuropsychiatric mechanisms (Martins-de-Souza et al., 2009; Ross et al., 1997; Adibhatla & Hatcher, 2008). However, phospholipase A2 can also participate in neurogenesis processes (Talib et al., 2008).

The guanine nucleotide-binding protein (G proteins) was downregulated in the hippocampi of pilocarpine-induced epilepsy. This is an important finding considering the wide role of this protein in the signal transduction by hormones, neurotransmitters, chemokines, and autocrine and paracrine factors (Neves et al., 2002).

In another study using the lithium-pilocarpine model, we identified 24 proteins in the hippocampal samples of rats, but only 7 were differentially expressed compared to control rats; namely, 4 were upregulated and 3 were downregulated (Marques-Carneiro et al., 2017). The interactome analysis revealed that the proteins are mainly related to glycolysis (14%) and to inflammation processes mediated by chemokine and cytokine signaling (5%). We also found proteins associated with Huntington's (5%) and Parkinson's disease (5%) and associated with fructose and galactose metabolism (4.80%). In addition, minor changes (2%) were also observed in several other pathways (Marques-Carneiro et al., 2017).

Two genetic models of absence epilepsy, GAERS and WAG/Rij, are resistant to the progression of partial seizures induced by electrical stimulation of the amygdala (Aker et al., 2010; Onat et al., 2007), hippocampus (Akman et al., 2008) or perirhinal cortex (Akman et al., 2010). Considering that absence seizures start in the thalamocortical circuitry, these data suggest an interaction between thalamocortical loop and limbic circuitry (Danover et al., 1998). Daniş et al. (2011) performed a 2D-PAGE to compare GAERS to Non-Epileptic Control (NEC) rats and showed six differentially expressed proteins, two in the parietal cortex (ATP synthase subunit delta and the 14-3-3 zeta isoform), two in the thalamus (myelin basic protein – MBP and macrophage migration inhibitory factor – MIF), and two in the hippocampus (MIF and 0-beta 2 globulin). Almost all proteins were upregulated in GAERS compared to NEC, except 0-beta globulin, a protein from hemoglobin complex predicted to be involved in oxygen transport (<https://www.ncbi.nlm.nih.gov/protein/CAA47877.1/>; GenBank: CAA47877.1). In line with these authors, MIF was also found upregulated in the frontal cortex and in the hippocampus of rats subjected to kainic acid-induced epilepsy (Lo et al., 2010). MIF, a pro-inflammatory cytokine released in response to inflammatory stimuli, is highly expressed in immune and nonimmune cells, including those in the brain. A study by Chai et al. (2020) showed that MIF is important to the process of hippocampal neurogenesis, affecting cell proliferation in the *dentate gyrus*.

Proteomics Profile of the Patients with Epilepsy

The study of biomarkers using proteomics in patients with epilepsy is a challenge. Access to the brain tissue consists of an essential approach to measure molecular dynamics at a determined point in the time course of the disease. However, in humans, the availability of brain tissue is very difficult; in epilepsy the brain is only available postmortem or from surgery performed on patients with focal epilepsy who do not respond to the pharmacological treatment (Do Canto et al., 2021). There are few studies using proteomics in humans, but compared to the last 10 years, we have seen a growing increase in data obtained from brain tissue and plasma and cerebrospinal fluid.

Some authors employed proteomics analysis to identify proteins differentially expressed in the hippocampi of patients with MTLE compared to control tissue obtained at autopsy (Yang et al., 2004). They found a reduction in the expression of the cytosolic enzyme acyl-CoA thioester hydrolase, known for its role in energy production through beta-oxidation in the mitochondria and peroxisomes, signal transduction, and protein kinase C activation, has been reported in patients with MTLE (Yang et al., 2004). In a subsequent study, the authors observed a reduction in the expression of 18 proteins playing different roles in the brain (Yang et al., 2006). Proteins with different roles as chaperone (TCP-1-alpha and HSP70), cell signaling (MAPKK), transcriptional signaling (NAD-dependent deacetylase sirtuin-2), or which are components of synaptosomes (synaptotagmin I and alpha-synuclein) and cytoskeleton (tubulins, vinculin, and profilin) are among them. On the other hand, increased expression of proteins associated with antioxidant function (peroxiredoxin 6), gliosis, and increased microvascular endothelial cells (apo A-I) was also reported by the same authors (Yang et al., 2005, 2006).

With the aim of obtaining biomarkers for TLE, Xiao et al. (2009) analyzed the cerebrospinal fluid (CSF) of patients using proteomics. The authors found five differentially expressed proteins in TLE patients compared to control, and six expressed only in patients with epilepsy. Vitamin D-binding protein (DBP) was increased, whereas cathepsin D, apolipoprotein J, Fam3c, and superoxide dismutase 1 (SOD1) were decreased in the TLE samples compared to the control. The six proteins found only in the patients were: tetranectin (TN), talin-2, apolipoprotein E, immunoglobulin lambda light chain (IGLc), immunoglobulin kappa variable light chain 1–5 (IGKV1–5), and procollagen C-endopeptidase enhancer 1 (PCOLCE). Table 15.2 shows the main functions of these proteins. Abnormal expression of some of these proteins, such as cathepsin D and SOD1, for example, has been reported in a study with proteomics using brain tissue (Eun et al., 2004), indicating that low levels of these proteins in the CSF may reflect deficiency in the brain (Xiao et al., 2009).

Despite most biomarkers to predict DR-TLE having been obtained mainly by samples of patients, the CSF has emerged as a promising source for the identification of brain biomarkers, since it is the body fluid with the closest anatomical contact with the changes that occur in the brain. Xiao et al. (2009) reported that the four proteins downregulated in the CSF of patients with DRE are proteins involved in

Table 15.2 Proteins differentially expressed in the CSF of patients with temporal lobe epilepsy and their main functions

Proteins downregulated in TLE	Function	References
Cathepsin D	Proteolytic processes; apoptosis; remodeling; inflammation.	Uchiyama et al. (2009)
Apolipoprotein J/Clusterin	Protective effect in response to brain injury; membrane recycling; lipid transportation; cell proliferation and death; neurodegeneration; pro- and anti-apoptosis.	Dragunow et al. (1995), Fritz et al. (1983), Kim and Choi (2011), May and Finch (1992), Wiggins et al. (2003), Zhang et al. (2005)
SOD1	Antioxidant enzyme; defense against oxygen toxicity.	Costello and Delanty (2004), Cardenas-Rodriguez et al. (2013)
Fam3c	Regulation of immune and inflammatory process in cancer; autophagy.	Hasegawa et al. (2014), Kraya et al. (2015)
Protein only detected in TLE	Function	References
Tetranectin (TN)	Glycoprotein and C-type lectin might be involved with tissue remodeling, mineralization during osteogenesis, migration of tumor cells, angiogenesis, and proteolytic process.	Wang et al. (2010), Dahiya et al. (2017)
Talin-2	Present at synapses, Talin-2 has an important role in cell adhesion and synaptic formation, mossy fiber sprouting formation.	Xiao et al. (2009, 2010)
Apolipoprotein E	Genetic factors involved in the mechanisms that might influence the age at onset of TLE (ApoE ϵ 4 isoform); neuronal death.	Salzmann et al. (2008), Kauffman et al. (2010)
Immunoglobulin lambda light chain (IGLc)	Interaction between the central nervous system and the immune system.	Haraldsson et al. (1992), Lischka et al. (1994)
Immunoglobulin kappa variable light chain 1–5 (IGKV1–5)	Interaction between the central nervous system and the immune system.	Haraldsson et al. (1992), Lischka et al. (1994)
Procollagen C-endopeptidase enhancer 1 (PCOLCE)	Extracellular matrix protein involved with the enhancement of procollagen C-proteinases; collagen metabolism.	Veznedaroglu et al. (2002), Xiao et al. (2009)

Data obtained from the study by Xiao et al., 2009

anti-inflammatory mechanisms, anti-oxidative, and neuroprotection, suggesting a possible role in the epileptogenesis (Table 15.2).

Cathepsin D belongs to the pepsin family of proteases and is one of the most studied aspartic proteases. This lysosomal protease is involved in proteolytic degradation, cell invasion, and apoptosis. Cathepsin D participates in the mechanism of

autophagy, a cellular process undertaken by neurons in the central nervous system to transport unneeded constituents to lysosomes (Uchiyama et al., 2009). This process is essential for the maintenance of cellular metabolism under physiological conditions. Processes that cause the reduction of lysosomal proteinases such as cathepsin D, B, and L, can induce neurodegeneration and be involved in epilepsy (Uchiyama et al., 2009).

Besides cathepsin D, downregulation of the antiapoptotic clusterin (apolipoprotein J) was also observed in the CSF of the patients with DRE compared to the controls (Xiao et al., 2009). These data are in line with those of the other authors, which showed reduced levels of CSF-clusterin in patients with drug-resistant and drug-responsive TLE compared to the controls, suggesting a pro-epileptogenic role (Yu et al., 2014). The clusterin, described as a glycoprotein, is presented as a nuclear form (nCLU) and a secretory form (sCLU) and perform dual role on apoptosis. nCLU is proapoptotic (Kim et al., 2012) and sCLU is antiapoptotic (Zhang et al., 2005). Clusterin is involved in several neurological diseases including Alzheimer's disease, Parkinson's disease (Milinkeviciute & Green, 2023; Charnay et al., 2012), and seizure-induced neuronal loss (Yu et al., 2014; Kim et al., 2012). Moreover, clusterin was markedly decreased in the CSF of patients with DRE rather than in patients with drug-responsive epilepsy (Xiao et al., 2009). The decreased level of CSF-clusterin in patients with DRE suggests that the cytoprotective effect of sCLU was attenuated in response to seizures. According to Yu et al. (2014), patients with DRE usually have a significant longer duration of disease compared to patients with drug-responsive epilepsy, suggesting that CSF-clusterin concentration could also be correlated with seizure and disease duration. Furthermore, time-dependent alteration of clusterin was also observed in the epilepsy model induced by kainic acid in rats (Kim et al., 2012).

Superoxide dismutase (SOD), a key antioxidant enzyme, cleans the superoxide radical and forms the first line of defense against oxidative stress and its subsequent effects (Costello & Delanty, 2004). Prolonged seizures may result in mitochondrial dysfunction, increased production of reactive oxygen species and nitric oxide, and neuronal cell death. Moreover, chronic mitochondrial oxidative stress and the resultant cellular dysfunction can render the brain more susceptible to epileptic seizures (Costello & Delanty, 2004). Thus, mitochondrial oxidative stress is considered a key factor in epileptogenesis. As a response to seizures, the synthesis of SOD1 protein protects against neuronal degeneration induced by oxidative stress, but hippocampal CA1 and CA3 neurons in an epileptic rat model no longer have SOD1 immunoreactivity (Kim et al., 2000). However, a reduced level of SOD was observed in the cortex of patients with TLE who had received surgical treatment (Eun et al., 2004) and in a rat model of electroconvulsive shock and pilocarpine (Erakovic et al., 2000; Bellissimo et al., 2001). Taking this into account, a lower level of SOD1 in the CSF of TLE patients described by Xiao et al. (2009) may reflect deficiency of antioxidant enzymes in the central nervous system in this pathological state.

FAM3C is a member of a cytokine-like gene family. Fam3c, also known as interleukin-like epithelial–mesenchymal transition inducer (ILEI), is a secreted protein found to be ubiquitously expressed in tissues, including bone, muscle, and brain

whose function has not been well known (Kraya et al., 2015). Studies have shown that the level of secreted Fam3c is markedly decreased in the brains of AD patients, whereas this protein destabilizes the β -secretase-cleaved APP carboxy-terminal fragment (Hasegawa et al., 2014). Overexpression of Fam3C significantly reduces the brain A β burden and ameliorates the memory deficit in AD model mice. Fam3c may be a plausible target for the development of disease-modifying therapies (Hasegawa et al., 2014). Otherwise, Fam3c has been reported as a member of a group of secreted proteins involved with tumor formation and metastasis (Kraya et al., 2015). High level of Fam3c has been detected in melanoma cell-conditioned media and patient, suggesting high level of autophagy associated with malignant melanoma's progression and aggressiveness (Kraya et al., 2015). On the other hand, the function of Fam3c in epilepsy has not been well determined.

The six proteins detected only in the CSF of patients with DRE might be involved in epileptogenesis (Xiao et al., 2009). Tetranectin is a glycoprotein which is encoded by the CLEC3B gene (C-type lectin domain family 3, member B) in human (Clemmensen et al., 1986). This protein is expressed in a variety of cells including monocytes, neutrophils, fibroblasts, and osteoblasts, and has been associated with the regulation of fibrinolysis, tissue remodeling, and proteolytic processes (Wang et al., 2010; Dahiya et al., 2017). Studies have shown that tetranectin has a defined role in the pathophysiology of epilepsy (Dahiya et al., 2017). Tetranectin levels were significantly downregulated in patients with DR epilepsy; the lower plasma tetranectin level was correlated with frequency of seizures and disease advances.

Talin-2 is another protein detected in CSF of patients with DRE. Talin-2 is a cytoskeletal protein found in high concentrations in the synapses. This protein plays an important role in the synaptic vesicle endocytosis, as in cell adhesion and in the recycling of synaptic vesicles (Xiao et al., 2010). To maintain continuous and reliable neurotransmitter release, synaptic vesicles must be rapidly recycled. A predominant mechanism responsible for synaptic recycling is clathrin-mediated endocytosis. Reduced levels of Talin-2 in the CSF may indicate impairment in the clathrin-mediated synaptic vesicle endocytosis, which can disturb neurotransmitter release and contribute to the epileptogenesis (Zheng et al., 2010).

Apolipoproteins play a well-established role in the transport and metabolism of lipids within the CNS, with a fundamental role in the maintenance and repair of neuron cell membranes (Mahley, 1988). The ApoE gene is polymorphic, and three isoforms have been identified ϵ 2, ϵ 3, and ϵ 4, showing different functions in the CNS (Morrow et al., 2000). Previous studies investigated the role of ApoE variants in TLE (Briellmann et al., 2000, Xu et al., 2021, Salzmann et al., 2008). Specifically, the results concerning the role of the ApoE ϵ 4 isoform as a modifier of the age of onset of epilepsy have been controversial (Kilpatrick et al., 1996, Tan et al., 2004). Kauffman et al. (2010) performed a molecular epidemiology study, a systematic review, and a meta-analysis to study the role of ApoE ϵ 4 isoform as a modifier of the age at onset of TLE. The authors showed that the ApoE ϵ 4 allele is associated with an earlier onset of TLE. These results were also confirmed by a meta-analysis study inasmuch all the populations analyzed showed a trend for a lower age at the onset of epilepsy in ApoE ϵ 4 carriers compared with noncarriers (Kauffman et al., 2010).

TLE, the most common form of partial epilepsy, is a polygenic and complex disease. There are several factors that might influence the age at the onset of TLE, and the genetic factor ApoE ϵ 4 seems to participate in some of them, such as, for example, disruption of the cytoskeleton, potentiation of apoptosis and increase in the deposit of β amyloid. Therefore, the findings highlight the ApoE gene as a candidate to influence the epileptogenic processes occurring in TLE. However, the authors reported that other variables that were not fully analyzed such as age, ethnicity, gender, and epilepsy etiology may be a source of bias in the results.

The immunoglobulin kappa-free light chains (IGKV1-5) and lambda-free light chains (IGLC) were also found to be expressed in the CSF of patients with DR-TLE but not in the CSF of control (Xiao et al., 2009). Since the discovery that autoimmune mechanisms were able to induce epilepsy in animal models and that gamma globulin could be used to treat epilepsy encephalopathy, subclasses of immunoglobulins have been investigated in the epileptic process. Increased kappa/lambda ratio has been reported in the serum and in cerebrospinal fluid of patients with various neurological diseases such as multiple sclerosis, amyotrophic lateral sclerosis, polyneuropathy, viral encephalitis, and intractable epilepsy, suggesting that the examination of light immunoglobulin chains is a good tool for diagnosing DR epilepsy in childhood (Bollengier et al., 1978; Lischka et al., 1994). In contrast, Haraldsson et al. (1992) observed a decreased serum total kappa/lambda ratio suggesting disturbed immunological mechanisms in children with DR epilepsy resulting from a developmental delay in immunopathophysiological and neuropathophysiological mechanisms in childhood epilepsies.

The final protein found by Xiao et al. (2009) expressed only in the CSF of patients with DR-TLE was the PCOLCE, an extracellular matrix protein that enhances the activities of procollagen C-proteinases (Table 15.2). This protein has also been found to be expressed in brain tissues (Takahara et al., 1994). PCOLCE is critical for collagen deposition. Studies in animal models have shown that, due to the chemoattractant property of collagen, this protein may play a role in cell migration and seizure activity in patients with TLE (Veznedaroglu et al., 2002). Therefore, Xiao et al. (2009) proposed that the presence of PCOLCE in the CSF of patients with TLE indicates cellular pathological changes in collagen metabolism in the epileptic brain.

A recent study performed to analyze the protein profile of patients with focal seizures (FS) due to TLE and with psychogenic nonepileptic seizures (PNES) showed that blood-brain barrier (BBB) damage is the main event that distinguishes patients with PNES from patients with FS (Hamrah et al., 2020). Under this condition of increased permeability of the BBB, molecules that are normally expected to be found only in the central nervous system, may diffuse into peripheral blood as well as serum proteins may reach the brain parenchyma. The altered proteins found in the serum following focal seizures were S-100B, ceruloplasmin, alpha 1-acilglycoprotein 1, and malate dehydrogenase 2, and they can be used as markers to differentiate seizures (Hamrah et al., 2020).

A study carried out by our group also showed the presence of serum albumin in hippocampal samples from patients with MTLE (Persike et al., 2018). Using

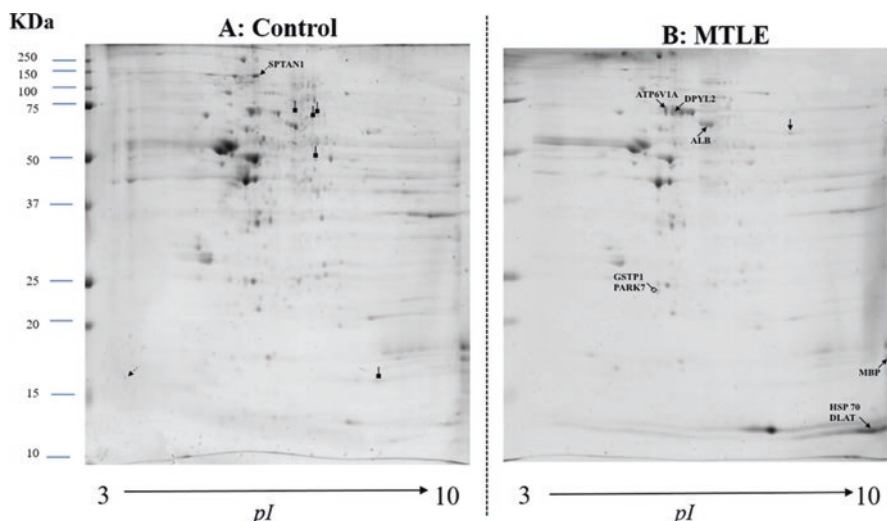


Fig. 15.2 Representative 2D-PAGE image of hippocampal protein extracts from control (a) and patients with mesial temporal lobe epilepsy (b). An amount (0.5 mg) of total protein of each sample was separated by isoelectrofocusing on a pH 3–10 linear gradient followed by the second dimension run on SDS-PAGE. Gels were stained with Coomassie brilliant blue. The peptides obtained from protein digestion of spots differentially expressed were analyzed by LC-ESI-MS/MS

proteomics (2D-PAGE coupled to LC-ESI-MS/MS) we have shown that the total number of spots was noticeably smaller in the hippocampus of MTLE patients with DRE than in control tissue (Fig. 15.2). A total of 16 proteins were differentially expressed in the hippocampal samples of patients with MTLE compared to control, but only 9 proteins were identified by NCBI database, as shown in Table 15.3. Among the nine identified proteins, six were upregulated, and one was downregulated in the hippocampal samples of MTLE group compared to control; two proteins were expressed only in the 2D-PAGE of patients with epilepsy. The identified proteins were: isoform 1 of serum albumin (ALB1), HSP70, dihydropyrimidinase-related protein 2 (DPYSL2), isoforms of myelin basic protein-1 (MBP1), isoform 3 of spectrin alpha chain (SPTAN1), proton ATPase catalytic subunit A (ATP6V1A), glutathione S-transferase P (GSTP1), protein DJ-1 (PARK7), and dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (DLAT). Glutathione S-transferase P and PARK7 were detected only in hippocampal samples of patients with MTLE. The expression of spectrin was downregulated in the hippocampi of the patients. All other proteins were upregulated in epilepsy. The expression of HSP-70, ATP6V1A and GSTP1 was validated by Western blot (Persike et al., 2018).

Most of the identified proteins do not possess a well-defined function in the epileptic process. However, some authors suggest that the increase in the expression of

Table 15.3 Proteins differentially expressed in the hippocampal samples of patients with MTLE

Gene symbol	Protein name	Changes	IP	MW
ALB	Isoform 1 of Serum albumin	↑	5.92	71,317
HSPA2	Heat shock-related 70 kDa protein 2	↑	5.56	70,263
DPYSL2	Dihydropyrimidinase-related protein 2	↑	8.2	77,912
MBP	Isoform 1 of Myelin basic protein	↑	9.79	33,097
SPTAN1	Isoform 3 of Spectrin alpha chain, brain	↓	5.21	282,906
ATP6V1A	V-type proton ATPase catalytic subunit A	↑	5.35	68,660
GSTP1	Glutathione S-transferase P	+	5.43	23,569
PARK7	Protein DJ-1	+	6.33	20,050
DLAT	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	↑	7.96	69,466

Proteins identified by LC-ESI-MS/MS and peptide matched by using Mascot MS/MS ion search and NCBI protein database. (Filled up arrow) upregulated proteins, (Open down arrow) downregulated proteins (MTLE versus control), and (+) proteins expressed only in the hippocampus of MTLE patients

myelin basic protein and albumin, may, for example, be indicative of alteration in the permeability of the blood-brain barrier and in myelination processes in experimental model of epilepsy (Huang et al., 2008; Marchi et al., 2010). Our data corroborate those of authors who show increased barrier permeability as a factor that contributes to epileptogenesis by facilitating the exposure of neurons to pro-inflammatory cytokines and predisposing them to drug resistance epilepsy.

The vacuolar H⁺ATPase, an evolutionarily ancient enzyme involved in neurotransmitter release mechanism (Wilkins et al., 2005) and acidification of synaptic vesicles after exocytosis (Li et al., 2005), was found to be upregulated in patients with MTLE compared to control. Likewise, the increased expression of HSP70 in hippocampal samples of the patients with epilepsy may represent a compensatory mechanism since HSP70 is a chaperone involved in the folding of new proteins (Mayer & Bukau, 2005).

The dihydropyrimidinase-related protein 2 (DPYSL2) is a member of cytosolic phosphoproteins, and is involved with neuronal migration, axon growth, and guidance (Morimura et al., 2013). Previous studies have reported an upregulation of DPYSL2 in hippocampal samples of patients with TLE (Persike et al., 2018) and in experimental model of epilepsy induced by pilocarpine (Marques-Carneiro et al., 2017). Increased expression of this protein has been associated with susceptibility to psychiatric disorders, such as schizophrenia (Ujike et al., 2006) or in diseases in which the psychiatric disorders appear as comorbidity such as Alzheimer's disease (Castegna et al., 2002). Upregulation of DPYSL2 was also observed in the hippocampi of patients with MTLE and considering its role in axonal growth and guidance, Persike et al. (2018) suggested that DPYSL2 can be involved in neuronal sprouting and seizures generation.

In contrast, the authors found downregulation of the spectrin protein in hippocampal samples of patients with MTLE (Persike et al., 2018). This protein is

responsible for anchoring the NMDA receptors (NR2A, NR2B, and NR1) to the actin cytoskeleton in the membrane, and it plays an important role in synaptic plasticity and long-term potentiation (LTP) (Wechsler & Teichberg, 1998). Thus, changes in spectrin protein expression can cause LTP impairment and cognitive deficit.

The glutathione S-transferase P (GSTP1) and PARK-7 were expressed only in the hippocampal samples of patients with MTLE. Previous study showed that these proteins play an important role as antioxidants following brain injury (Sharma et al., 2004), in addition to playing an important role in detoxification. Besides, GSTP1 has been associated with the inactivation of antiepileptic drugs in the liver (Shang et al., 2008), and it may be responsible for the poor penetration of antiseizure medications. This protein may represent an important target for studies related to drug resistance frequently associated with MTLE. On the other hand, upregulation of PARK-7 indicates the presence of neuroprotective mechanisms associated to MTLE (Persike et al., 2018).

Mériaux et al. (2014), using MALDI mass spectrometry imaging (MSI) coupled with proteomics found seven neuropeptides differentially expressed in the layer of dentate gyrus (DG) of patients with MTLE. The identified proteins exert different roles being associated with axons regeneration (neurotrophin), extracellular matrix proteins, cell surface proteins, membrane proteins, G proteins, cytoskeleton proteins, and tumor suppressors (TS). The Leucine-rich glioma inactivated 1 (LGI1) protein was found in the hippocampus of MTLE and has been related to the heritability of TLE.

A proteomic study performed by Keren-Aviram et al. (2018) investigated proteins differentially expressed in the neocortex of patients with DRE, at high-frequency spikes. The authors identified eight upregulated proteins (SNCA, STMN1, UGP2, DSP, CA1, PRDX2, SYN2, and DPYSL2), and ten downregulated (GFAP, HNRNPK, CPNE6, CRYAB, GNAO1, PHYHIP, HNRPDL, ALDH2, GAPDH, and LASP1). In their analysis, the authors associated proteins with vascular modifications and the decreased expression of GFAP α as being related to metabolic changes and increased cortical activity.

Proteomics studies using plasma are very scarce. Banote et al. (2021) identified 41 proteins differentially expressed compared to the control group. Some of them have previously been reported to be associated with epilepsy, including Pentraxin-related protein (PTX3), von Willebrand factor (VWF), Sonic hedgehog (SHH), MAPK14, a member of mitogen-activated protein kinases, Apolipoprotein E (APOE), C-C motif chemokine 5 (CCL5), Laminin subunit beta-2 (LAMB2), and FYN-binding protein 1 (FYB1). Many of these proteins are involved in epileptogenic processes, such as inflammation, calcium ion binding, lipid binding activity, metal ion binding, and identical protein binding activity.

The presence of the chemokine CCL5 has also been reported by Toledo et al. (2021) in the cerebral cortex of patients with drug-resistant TLE (DR-TLE) compared to patients with TLE responders to treatment and healthy controls. According to the authors, the presence of CCL5 together with other cytokines (IFN- γ

and IL-17) in the cerebral cortex of the patients indicates involvement in lymphocyte recruitment, suggesting communication between central and peripheral inflammatory markers (Toledo et al., 2021).

The endogenous level of CCL5 is very low in the plasma of healthy individuals, but it increases dramatically in both peripheral and CNS in pathological conditions, i.e., in autoimmune diseases (encephalomyelitis and multiple sclerosis), and in DR-TLE (Pittaluga, 2017; Toledo et al., 2021; Wolinski et al., 2022). There are two mechanisms by which the CCL5 can become bioavailable in the CNS, (i) by the permeabilization of the blood-brain barrier during inflammation, which favors the entry of CCL5 from the periphery into the brain, (ii) by concomitant massive local production of CCL5 from activated astrocytes and, to a lesser extent, from microglial cells triggered by pro-inflammatory cytokines (Pittaluga, 2017). Once released in the synaptic cleft, CCL5 regulates the function of glial cells (microglia and astrocytes) themselves through autocrine processes by means of CCR5 receptors, and to a less extent, of CCR1 and CCR3 receptors (Pittaluga, 2017). In physiological conditions, the expression of CCR5 in astrocytes is low but rapidly increases upon stimulation by cytokines released by microglial cells contributing to higher expression of CCR proteins (Sørensen et al., 1999). Thus, this cascade is an event related to neuroinflammation.

In CNS, CCL5 is involved with Ca^{++} ions mobilization, suggesting that this chemokine could modulate glutamate exocytosis in the CNS (Meucci et al., 1998; Klein et al., 1999; Musante et al., 2008; Pittaluga, 2017). However, some authors have shown that the CCL5-induced changes to glutamate release are area specific and involve different receptor repertoires, indicating heterogeneity in the effect of this chemokine in the CNS (Di Prisco et al., 2012). CCL5 plays a role in coupling inflammation and synaptic excitability in CNS diseases secondary to viral infections, such as acquired immune deficiency syndrome-related dementia, or involving neuroinflammatory processes, such as MS and Alzheimer's dementia (Pittaluga, 2017).

When we compared the proteins differentially expressed in the hippocampus of patients with MTLE and from rats subjected to pilocarpine compared to their respective controls, we identified three proteins in common, Park-7, DPYSL2, and ATP6V1A (Table 15.3). The proteins DPYSL2 and ATP6V1a were differentially increased in both patients, and rat hippocampi, while Park-7 was differentially expressed in rat (increased expression compared to control) and found exclusively expressed in the samples of MTLE patients. Increased expression of these proteins may be involved in neuronal development and plasticity (DPYSL2), neuroprotection mechanisms (Park-7), and neuronal excitability (ATP6V1a). The ATP6V1a is a protein involved with releasing neurotransmitters and acidifying synaptic vesicles after exocytosis for recycling (Li et al., 2005; Wilkens et al., 2005). The upregulation of this protein can reflect an increase in the dynamics of synthesis, storage, and release of neurotransmitters present in epileptic tissue and therefore increased excitation (Marques-Carneiro et al., 2017; Persike et al., 2018) (Table 15.4).

Table 15.4 Proteins differentially expressed in patients and in rats with MTLE

Gene symbol	Protein name	Changes in Pilocarpine model x control	Changes in epileptic human hippocampus x control
Park7	Protein DJ-1	↑	+
Dpysl2	Dihydropyrimidinase-related protein 2	↑	↑
Atp6v1a	V-type proton ATPase catalytic subunit A	↑	↑

Proteins differentially expressed in the hippocampal samples of rats subjected to pilocarpine (90 days following SE), and of patients with MTLE. (Filled up arrow) upregulated proteins and (+) protein expressed only in the hippocampus of the patients (Persike et al., 2012, 2018)

15.2 Conclusions

Proteomics has contributed to improve knowledge about altered mechanisms in the hippocampus (the main area affected in this disease) of patients with TLE, and experimental models of TLE. Proteomics, a powerful methodology that combines ancient techniques (i.e., two-dimensional electrophoresis and amino acid analysis) with advanced technologies (mass spectrometry), emerges as a powerful alternative in the search for target proteins to treat or perhaps prevent the occurrence of seizures. Proteomics allows not only detects the differential expression of proteins in the hippocampi of patients with epilepsy in relation to the control tissue but also provides information about the protein class to which they belong, the cellular compartment where they are found, and their molecular and biological functions, through qualitative analysis using specific software (GENEmania system, Metacore software) (Marques-Carneiro et al., 2017; Persike et al., 2018; Canto et al., 2022).

Data from studies with proteomics in the hippocampus of patients with MTLE and from experimental models of MTLE show common mechanisms involved with synaptic plasticity, neurotransmission, and neuroprotection. The proteins involved in these mechanisms may be important biomarkers for studies of these alterations associated with the epileptic condition.

Considering the complex physiology of the central nervous system, it may be convenient to combine simultaneous analyzes of material collected by surgical procedure from patients with MTLE. Patients with adequate clinical follow-up undergoing surgical treatment of epilepsy can provide important material for the research of biomarkers using proteomics.

Unbiased big data mining has emerged to generate patterns of genes, proteins, and metabolites, and signaling pathways, from brain tissue samples from MTLE patients (Kirchner et al., 2020). The generated data set can be organized into a multivariate interactome, together with clinical data, in the search for biomarkers or therapeutic targets employing experimental models or in vitro preparations in reverse translation. Currently, proteomics studies have been greatly relying on the mass spectrum (MS). Despite the high sensitivity, molecules with low

concentrations in the tissue may not be detected. Thus, neuroscience still needs more sensitive methods that can directly read entire protein sequences without having to resort to databases to identify theoretical proteins.

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Chapter 16

GABAergic Neurotransmission

Abnormalities in Pharmacoresistant Epilepsy: Experimental and Human Studies



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Abstract Despite the fact that there are several drugs available for the treatment of epilepsy, pharmacoresistance remains a major challenge in seizure control. Therefore, a significant part of epilepsy research has focused on revealing the mechanisms underlying drug resistance in order to develop new rationally designed pharmaceutical therapies for refractory epilepsies. Based on experimental and clinical studies, epilepsy-induced structural and functional alterations in brain targets have been postulated to lead to decreased sensitivity to antiepileptic drugs, more recently antiseizure medications (ASM). Also, evidence shows that GABA neurotransmission system plays a leading role in the pathophysiology of epilepsy. Canonically, GABA (gamma-aminobutyric acid) is considered the main inhibitory neurotransmitter in the central nervous system, but due to the variability in the location and composition of its receptors by different types of subunits, as well as neural physiological immaturity, GABA may have excitatory effects. Abnormalities in the GABAergic system identified in animal models of epilepsy and in samples of brain tissue samples resected surgically from patients with drug-resistant epilepsy, mainly at level of GABA_A receptors (GABA_ARs), whose changes lead to an altered response to some ASMs, among other neuroleptic drugs.

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Here we review the current evidence on changes in the GABAergic system related to seizure generation, epilepsy, and pharmacoresistance, with particular emphasis on GABA_ARs and genetic polymorphisms of its subunits associated with refractory human epilepsy.

Keywords Pharmacoresistant epilepsy · GABA neurotransmission · GABA · Receptors · Human data · Animal models · GABA genetics · Antiseizure medication

16.1 Introduction

Epidemiological data indicate that 20–40% of patients with epilepsy are refractory to treatment with antiepileptic drugs or rather antiseizure medication (ASM). Based on experimental and clinical studies, neurobiological theories have been proposed to explain the drug-resistant epilepsy: (a) the “multidrug transporter hypothesis” suggests that increased brain expression of drug efflux transporters, such as P-glycoprotein (P-gp), reduces ASM levels in the brain, and (b) the “target hypothesis” postulates that acquired (epilepsy-induced) structural and functional alterations in brain targets lead to diminished sensitivity to ASMs (Remy and Beck 2006). Antiseizure activity, commonly depends on the interaction of a drug with one or more molecular targets in the brain. These targets include voltage-dependent ion channels, and molecules involved in neurotransmitter release, uptake, and metabolism, as well as neurotransmitter receptors (Rogawski et al. 2016; Remy and Beck 2006). Recently, (c) the “neuronal network hypothesis” proposes that the loss of neurons leads to remodeling of the synaptic network, altering the seizure control system in the brain. In addition, (d) the “intrinsic severity hypothesis” suggests that high seizure rates are an important predictor of refractory or drug-resistant epilepsy, and (e) the “genetic variant hypothesis” proposes that some gene polymorphisms are associated with changes in pharmacodynamics and pharmacokinetics of ASMs, metabolic pathways, enzymes, ion channels, and neurotransmitter receptors (Tang et al. 2017; Bazhanova et al. 2021). Indeed, it is likely that mechanisms related to all five hypotheses are involved in drug resistance.

It has been considered that seizures are caused by an imbalance between hypersynchronization and desynchronization. Gamma-aminobutyric acid (GABA) is considered the main inhibitory neurotransmitter in adulthood that exerts a control on excitatory activity. Indeed, several ASMs exert their effects by enhancing the inhibitory effect of GABA (Rogawski and Löscher 2004). Activation of GABA-type A receptors (GABA_ARs) induces a fast inhibition in the central nervous system (CNS). GABA_ARs mediate both phasic and tonic inhibition and are molecular targets for the action of numerous classes of drugs, including anxiolytics, ASMs, and sedative-hypnotics, such as benzodiazepines, barbiturates, alcohol, anesthetics, and

neurosteroids. Because GABA_ARs are ubiquitously expressed throughout the CNS, changes in their expression and function are implicated in virtually all aspects of brain function. Additionally, deficits in GABA_AR-mediated neurotransmission are implicated in diverse disorders of the CNS, such as epilepsy. This review focuses on the changes that have been detected in the various components of GABAergic neurotransmission with an emphasis on clinical and genetic studies.

16.2 GABAergic Neurotransmission

GABA neurotransmitter is synthesized via decarboxylation of glutamate by glutamic acid decarboxylase (GAD). The enzymatic activity of GAD is regulated by its expression and the degree of association with the cofactor pyridoxal phosphate (PLP) (Soghomonian and Martin 1998). There are two main isoforms of GAD, GAD65, and GAD67 (65 kDa and 67 kDa, respectively), each encoded by different genes (Erlander et al. 1991), and distinct catalytic and kinetic properties, as well as subcellular localization (Walls et al. 2010, 2011). After its release into the synaptic cleft, GABA is reuptaken by GAT-1 and GAT-3 into neurons and glial cells. GABA catabolism is carried out by GABA transaminase (GABA-T) that converts it to succinic semialdehyde (SSA) by transamination using glutamate and α -ketoglutarate (α KG) as cosubstrates. SSA is subsequently oxidized by SSA dehydrogenase (SSADH) to succinate, a constituent of the tricarboxylic acid cycle (TCA). Alternatively, SSA can be reduced by c-OH-butyric acid dehydrogenase (GHBDH) to c-OH-butyric acid, which can activate GABA_BRs (Kaupmann et al. 2003).

It is well known that GAD inhibition can produce seizures, whereas the expression of GAD antibodies is associated to encephalitis causes both acute seizures and chronic epilepsy with predominantly temporal lobe onset (Li et al. 2020). Because GAD is dependent on pyridoxal phosphate as the coenzyme, carbonyl trapping agents such as derivatives of hydrazine are generally convulsant in nature (Tapia 1975). Interestingly, aminooxyacetic acid exerts proconvulsant effects at high doses, while at lower doses, it is an anticonvulsant (Tapia 1975).

In the nerve terminals, GABA can be released to the synaptic cleft by two different pathways, i.e., via Ca²⁺-dependent vesicular release or Ca²⁺-independent release via transporter reversal (Belhage et al. 1993).

GABA released is transported back into the presynaptic nerve terminal or into surrounding astrocytes via a high-affinity GABA transport system, thereby terminating its action (Iversen and Kelly 1975). It has been estimated that, in contrast to the astrocytic GABA transport mechanism, neuronal GABA transport system is three- to sixfold more efficient which indicates a reutilization of the uptaken GABA (Hertz and Schousboe 1987). The degradation of GABA occurs via the enzymes GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase, yielding succinate. GABA-T is located in both neurons and astrocytes, with the highest activity in the latter cell type (Schousboe et al. 1983). Figure 16.1 illustrates a GABAergic synapse with its main components.

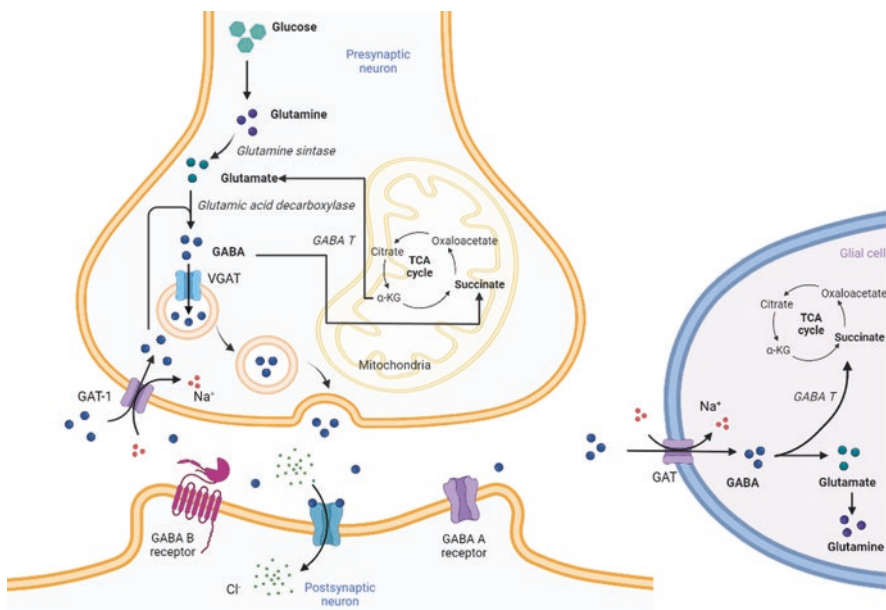


Fig. 16.1 Gamma-aminobutyric acid (GABA) is synthesized by decarboxylation of glutamate by glutamic acid decarboxylase (GAD). Upon release into the synaptic cleft, GABA can interact with receptors. To terminate this effect, the neurotransmitter is uptaken by high affinity membrane transporters into neurons and surrounding glia, where they can be recycled or metabolized via several enzymes. GABA is converted to glutamine in glial cells. The high activity of glutamine synthase metabolizes glutamic acid to glutamine and can be recycled to neurons to produce glutamate or GABA

GABA mediates its action via two classes of receptors, ionotropic GABA_ARs and GABA_CRs and metabotropic GABA_BRs. Unlike GABA_ARs and GABA_CRs, which act as ligand-gated Cl⁻ channels and are involved in fast synaptic inhibition, the GABA_BRs are guanine nucleotide binding (G) protein-coupled receptors that modulate calcium (Ca²⁺) and potassium (K⁺) channels and elicit both presynaptic and slow postsynaptic inhibition (Watanabe et al. 2002). The GABA_ARs are constituted from a family of 19 homologous genes categorized by degree of sequence identity as different subunit families (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) (Barnard et al. 1998; Olsen and Sieghart 2008; Olsen 2018). GABA_ARs, as heteropentameric complexes of subunits, form an integral anion channel permeable to chloride and bicarbonate ions (Fig. 16.2). In the brain, GABA_ARs are composed of two α subunits, which in turn are presented as six isoforms (α1, α2, α3, α4, α5, and α6), two β subunits present as three isoforms (β1, β2S, β2 L, and β3) that contribute to the binding site of GABA (Scimemi et al. 2005; Pirker et al. 2003), and one γ subunit with four isoforms (γ1, γ2S -γ2L, and γ3) (Buró and Kamatchi 1991; McKernan and Whiting 1996) (Fig. 16.2). This diversity of different combinations of isoforms and their assembly determines the properties of GABA_ARs, such as affinity for GABA, allosteric modulation, interaction with intracellular proteins,

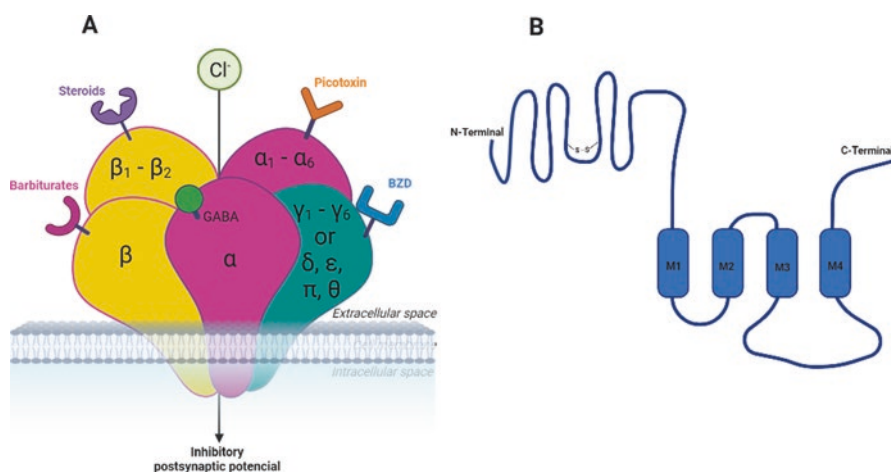


Fig. 16.2 (a) GABA_ARS structure. It is an ionotropic receptor type that comprises different subunits: α alpha subunit, β beta subunit, γ gamma subunit, δ delta subunit, ϵ epsilon subunit, π subunit phi, and θ theta subunit. Benzodiazepine-binding site (BZD), Cl^- chloride ion. (b) Each subunit comprises four transmembrane domains (M1–M4). The large intracellular loop between M3 and M4 domains contains consensus sites for phosphorylation by protein kinases. The M2 domain provides the lining of the Cl^- pore intrinsic to the pentameric structure. The most abundant GABA_AR in the brain is the $\alpha 1\beta 2\gamma 2$ pentamer (McKernan and Whiting 1996; Kaupmann et al. 1998)

probability of channel opening, kinetics, and conductance. Therefore, changes in the composition of the receptor subunits appear to affect GABAergic neurotransmission (Wang and Buzsaki 1996; Lambert et al. 2003).

GABA_BRs, broadly expressed in the nervous system, modulate synaptic excitability and plasticity in the cerebral cortex, rhythmic activity in cortico-thalamic circuits, primary afferent inputs to the spinal cord and brainstem, and the activity of dopaminergic and other monoaminergic neurons. GABA_BRs are implicated in a wide variety of neurologic and psychiatric disorders, including generalized and focal epileptic disorders, absence seizures and γ -hydroxybutyrate toxicity (Avoli and Lévesque 2022). Baclofen is the only clinically available GABA_BR agonist used for the treatment of spasticity, dystonia, and some types of neuropathic pain (Bormann 1988; Bowery 1989; Marshall et al. 1999). The GABA_BR, like other members of this class, is an obligatory heterodimer formed by subunits GABA_B1 and GABA_B2. The GABA_B1 subunit contains a large extracellular domain that binds GABA or other ligands, such as baclofen; the GABA_B2 subunit couples the receptor with the effector G protein (Bowery 1989). Activation of GABA_BRs results from successive conformational changes within and across its two subunits. The binding of GABA to the GABA_B1 subunit induces a relative movement of the extracellular domains of both GABA_B1 and GABA_B2, which elicits a conformational change of a transmembrane domain that activates the G protein (Fig. 16.3).

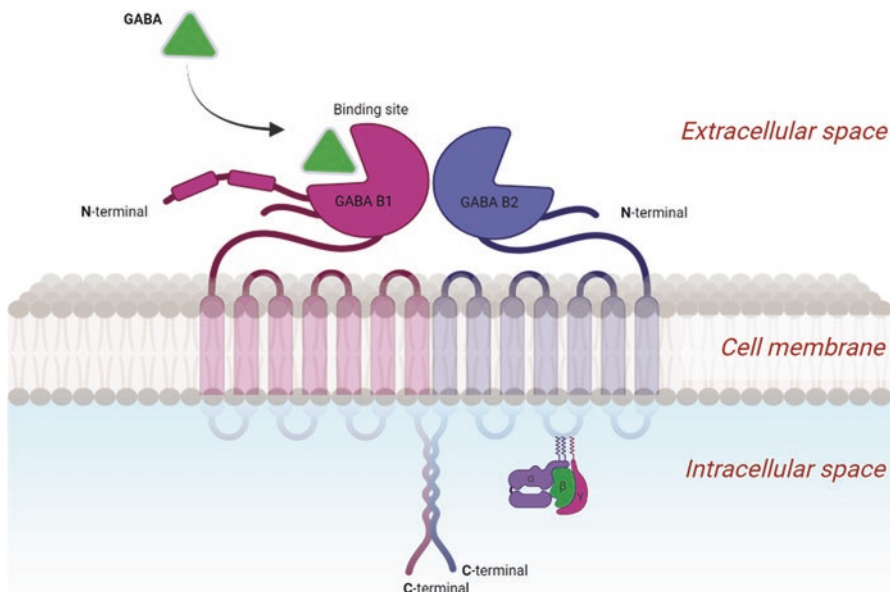


Fig. 16.3 The GABA_B receptor is a metabotropic receptor that acts through second messengers. GABA_B1 and GABA_B2 subunits form a functional heterodimer. Agonists bind to the N-terminal of the GABA_B1 subunit, whereas allosteric modulators bind to the GABA_B1 subunit, N amino terminal, C carboxyl terminal

There are two predominant isoforms of GABA_B1 subunits in the brain, which differ by the presence of a tandem pair of extracellular domains (sushi domains) in their amino (N)-terminal region in GABA_B1a isoform, which not exist in GABA_B1b. These domains are protein-binding motifs involved in protein–protein interactions and determine the different synaptic distributions and functions of the two isoforms of GABA_B1 subunits (Ulrich and Bettler 2007; Benarroch 2012).

The GABA analog cis-4-aminocrotonic acid (CACA) selectively activates a third class of GABA_ARs in the mammalian CNS. These receptors, which were tentatively designated GABA_CRs in 1984, are Cl⁻ pores insensitive to both bicuculline and baclofen (Johnston 1996). Several lines of evidence now indicate that GABA_CRs are composed of ρ -subunits (Bormann and Feigenspan 1995; Enz and Cutting 1998). Heterologously expressed, these subunits form homo-oligomeric channels with the characteristic GABA_CRs (Bormann and Feigenspan 1995; Feigenspan and Bormann 1998; Enz and Cutting 1998, 1999). GABA_CRs comprise the GABA ρ 1 subunit but eventually grow to a total of three subunits: GABA ρ 1, GABA ρ 2, and GABA ρ 3.

Currently, the name GABA_C is in disuse, and the three GABA ρ genes are included in the GABA_AR family (GABA_AR, ρ subunits) (Fig. 16.4). They are part of the Cys-loop superfamily of neurotransmitter receptors, also called the ligand-gated ion channel (LGIC), which includes GABA_ARs, nicotinic acetylcholine receptors

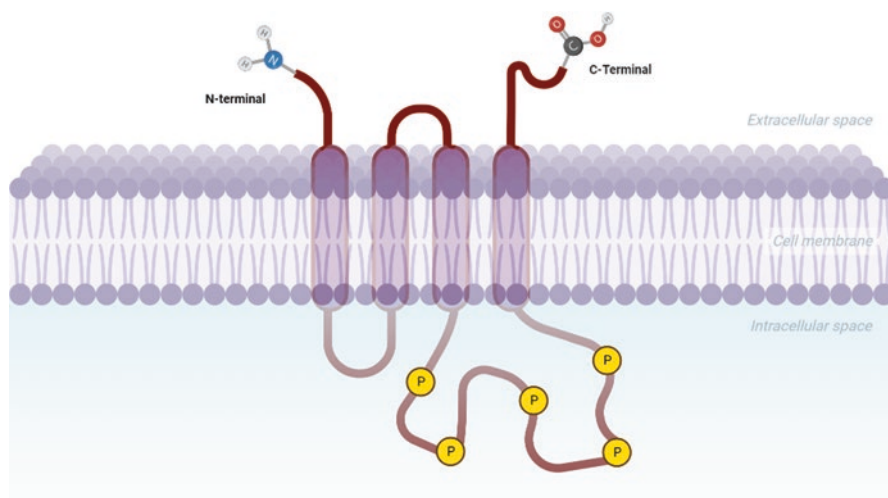


Fig. 16.4 Structure of rho subunits of GABA_ARs. The rho subunits structure comprises a long extracellular region, with N-terminal and two cysteine residues. It presents four domains that go across the cell membrane and end with a short C-terminal region. The second domain comprises the ionic channel of the receptor. The extracellular domain includes the GABA binding sites and other modulatory sites. N amino terminal, C carboxyl terminal; blue circle cysteine residues, P phosphorylation sites (McKernan and Whiting 1996)

(nAChR), glycine receptors, ionotropic serotonin receptors (5HT₃), and a Zn²⁺-activated ion channel (Olsen and Sieghart 2008).

Fast synaptic GABAergic transmission relies essentially on Cl⁻ fluxes through GABA_ARs (Farrant and Nusser 2005), and the maintenance of the electrochemical Cl⁻ gradient is essential to the GABA-mediated neuronal effects (Ben-Ari et al. 2012). Changes in neuronal Cl⁻ homeostasis affect GABA_AR-mediated transmission and may contribute to epileptic activities. In this sense, cation-Cl⁻ cotransporters (CCCs), studied initially for their role in the regulation of cellular volume, are now also considered targets for the control of the cellular electrochemical Cl⁻ gradient (Blaesse et al. 2009). The CCC family in mammals consists of nine members encoded by the genes Slc12a1-9 (Blaesse et al. 2009). CCCs are glycoproteins of 120–200 kDa, seven of which have been identified as plasmatic proteins (Mercado et al. 2004) with a predicted secondary structure of 12 membrane-spanning segments flanked by intracellularly located carboxyl and amino-terminal domains (Gerelsaikhon and Turner 2000). Functionally, CCCs are categorized into three groups: (1) two members cotransport Na⁺/K⁺/2Cl⁻ toward the inside of the cell and are named NKCC1 and NKCC2; (2) four members cotransport K⁺/Cl⁻ toward the outside of the cell and are named KCC1-4; and (3) one member cotransports Na⁺/Cl⁻ toward the inside of the cell and is named NCC. The physiological roles of proteins encoded by the Slc12a8 (CIP1) and Slc12a9 (CCC9) genes remain unknown (Mercado et al. 2004; Blaesse et al. 2009; Briggs and Galanopoulou 2011).

Except for NKCC2 and NCC, which are predominantly found in the kidney, all CCCs are expressed in neurons, glial cells, or both in specific patterns during the CNS development (Mercado et al. 2004; Blaesse et al. 2009). KCC2 is exclusively expressed in neurons (Payne et al. 1996; Rivera et al. 1999; Gulyas et al. 2001) and linked to NKCC1; both are responsible for maintaining the neuronal electrochemical Cl^- gradient (Ben-Ari et al. 2012). Interestingly, in immature neurons, the expression level of NKCC1 is higher when compared with KCC2. Then, in the immature brain, the intracellular Cl^- concentration is higher than the extracellular concentration, and GABA_AR activation induces membrane depolarization and neuronal excitation through Cl^- efflux. In mature neurons, the expression level of KCC2 is higher than NKCC1, and thus GABA_AR activation results in hyperpolarization and neuronal inhibition through Cl^- influx (Rivera et al. 1999; Gulyas et al. 2001; Dzhala et al. 2005; Briggs and Galanopoulou 2011; Antrobus et al. 2012; Ben-Ari et al. 2012).

16.3 Involvement of GABA_ARs in Seizure, Epilepsy, and Pharmacoresistance

16.3.1 GABA_ARs Expression in Experimental Models of Epilepsy

At least in part, pharmacoresistance in epilepsy has been related with changes in the properties of drug targets, such as changes in potential-dependent ion channels and neurotransmitter receptors (for example, the GABA and glutamate receptors) leading to a decrease in drug sensitivity, in both experimental models (this section) and human epilepsy patients (Sect. 16.3.2). In this sense, several ASMs lose their effectiveness when their effect depends on interaction with GABA_ARs (Remy and Beck 2006). In the pilocarpine-induced epilepsy model, clonazepam effects were significantly reduced in CA1 pyramidal neurons and lightly increased in dentate granule cells (DGCs) (Gibbs III et al. 1997). In contrast, valproate and phenobarbital did not modify spike-like activity of CA3 pyramidal neurons of animals with epilepsy (Klitgaard et al. 2003). Clonazepam ineffectiveness has been associated with a switch between α subunits of GABA_ARs, with the $\alpha 4$ subunit highly expressed in animals with epilepsy and replacing the $\alpha 1$ subunit (Brooks-Kayal et al. 1998).

In general, the benzodiazepine binding site of GABA_ARs is located at the interface between the α and γ subunits, and its pharmacology is thus influenced by these subunits (Fig. 16.2) (Ogris et al. 2004). Whereas the GABA_ARs with $\alpha 1$ –3 or $\alpha 5$ subunits have approximately the same affinity for classical benzodiazepines, but have differential affinity from nonclassical benzodiazepines, such as zolpidem, zaleplon, and abecarnil (Korpi et al. 2002; Ogris et al. 2004). The benzodiazepines insensitivity of GABA_ARs containing $\alpha 4$ and $\alpha 6$ subunits is due to the presence of

an arginine residue instead of a histidine in a conserved position in their binding site (residue 101) (Wieland et al. 1992). In kainite- and pilocarpine-induced epilepsy, the expression level of $\gamma 2$ and $\alpha 5$ subunits is modified in both the hippocampus and dentate gyrus (Schwarzer et al. 1997; Fritschy et al. 1999; Peng et al. 2004; Houser and Esclapez 2003). In addition, it has been suggested that CA1 pyramidal neurons might contain $\alpha 4$ and $\gamma 2$ subunits in its GABA_ARs in animals with epilepsy. In this sense, ultrastructural studies demonstrated that in the pilocarpine model, both the $\gamma 2$ and $\alpha 4$ subunits have similar perisynaptic locations (Zhang et al. 2007), but at some time points the $\alpha 4$ subunit expression is reduced (Lund et al. 2008). The presence of $\alpha 4\beta X\gamma 2$ GABA_ARs assemblies in principal neurons of epileptic hippocampus suggests increases in tonic inhibition, with less sensitivity to benzodiazepines and zinc, and changes in $\gamma 2$ subunit phosphorylation-mediated recruitment and trafficking (Farrant and Nusser 2005; Jacob et al. 2008; Leidenheimer 2008).

In general, GABA_ARs containing $\alpha 5$ subunit are positioned extrasynaptically and modulate NMDA receptors (Li et al. 2005). The low expression of these receptors in the epileptic hippocampus could augment NMDA excitation. The decreased expression of the GABA_AR δ subunit, which mediates tonic GABAergic inhibition in DGCs produces an increase in GABAergic inhibition (Nishimura et al. 2005; Zhang et al. 2007; Zhan and Nadler 2009), probably as consequence of a compensatory up-regulation of the extrasynaptic GABA_AR $\alpha 5$ subunit in DGCs (Fritschy et al. 1999). Consistent with the role of extrasynaptic GABA_ARs in epilepsy, mice deficient in the GABA_ARs δ subunit and GABA_ARs $\alpha 5$ exhibit increased seizure susceptibility (Mihalek et al. 1999; Glykys et al. 2009). Beyond the general decrease in the GABA_ARs δ subunit, a more specific decrease in the immunolabeling for this subunit has been detected in perisynaptic locations of DGCs following pilocarpine-induced status epilepticus. However, in agreement with previous studies, tonic inhibition in DGCs was maintained, while the phasic inhibition was decreased (Zhang et al. 2007). Moreover, δ subunit-containing receptors, which mediate tonic inhibition in DGCs, have higher neurosteroid sensitivity (Mihalek et al. 1999; Wohlfarth et al. 2002). Joshi et al. (2013) hypothesized that loss of neurosteroid sensitivity on DGCs increases their excitability. Additionally, it is proposed that tonic inhibition, mediated primarily by δ and $\alpha 5$ subunits, provides a potent shunt inhibition that keeps neuronal excitability in check modulating the offset (threshold) of the I/O curve (Stell et al. 2003; Pavlov et al. 2009). Thus, tonic conductance mediated by these receptors increases the excitatory drive needed to induce the action potential firing. The downregulation of these receptors in temporal lobe epilepsy (TLE) can contribute to enhanced excitability (Mihalek et al. 2001; Glykys and Mody 2006).

Changes in other GABA_ARs subunits are less consistent, reporting increments or reductions depending on the experimental epilepsy model, brain region, subcellular locations, or the methodological approach used (Brooks-Kayal et al. 1998; Laschet et al. 2007; Schwarzer et al. 1997). In summary, in cortical and limbic structures, decreased expression of $\alpha 1$, $\beta 2/3$, and $\gamma 2$ subunits, and increased expression of $\alpha 4$

and δ subunits are observed in both epileptic and ASM-resistant rats. These receptors exhibit a reduced response to GABA, benzodiazepines, barbiturates, and zinc, as well as modifications in plasmatic clustering, intracellular scaffolding, signaling, vesicular traffic, and recycling (Mele et al. 2019; Shi et al. 2019; Löscher et al. 2020). In addition, functional coupling of GABA_AR with other neurotransmitter receptors or ionic channel also may be altered (Marsden et al. 2007; Shen et al. 2016; Mele et al. 2019).

On the other hand, in early developmental stages of the CNS, GABA_ARs show low responsiveness to its agonists and positive modulator, which has been associated with the predominant expression of its $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\beta 3$ subunits. This condition change during development, and the $\alpha 1$ -2/2 $\beta 1/\gamma 2$ assembly for postsynaptic GABA_ARs becomes predominant (Barker and Hines 2020; Schipper et al. 2016). However, beyond the GABA_AR composition in subunits, its activation induces neuronal depolarization and neuronal excitation in immature brain (Ben-Ari et al. 2012). GABA-mediated excitation at early development stages has trophic effects on neural differentiation and migration, as well as circuit formation (Manent et al. 2006; Cancedda et al. 2007; Wang and Kriegstein 2010). In addition, it is involved in an increased seizure susceptibility (Jensen 2009; Briggs and Galanopoulou 2011) and glutamate-mediated excitotoxicity (Hilton et al. 2005). It is noteworthy that the GABA immaturity condition, where the expression level of NKCC1 is higher than KCC2, is also observed in pathological conditions such as human epilepsies (Muñoz et al. 2007; Bragin et al. 2009; Liu et al. 2020) and in preclinical models of epileptogenesis (kindling) (Mazarati et al. 2009) and epilepsy (pilocarpine) (Li et al. 2008). Therefore, the NKCC1 blockage with bumetanide was proposed as therapy in the treatment of neonatal seizures (Dzhala et al. 2005; Almeida et al. 2011; Löscher et al. 2013; Pressler and Auvin, 2013). However, preclinical research does not support the use of bumetanide as ASM because conflicting results and side effects were induced when bumetanide was tested in patients with drug-resistant TLE, seizures due to cortical malformations (Maa et al. 2007; Kahle and Staley 2009), and in neonatal seizures as adjunct to phenobarbital (Pressler et al. 2015). Various strategies to regulate the expression of KCC2 and NKCC1 with interesting, but still contradictory results have been assessed (Chen et al. 2017; Liu et al. 2020). As example, because brain-derived neurotrophic factor (BDNF) downregulates KCC2 expression, blockage of the neuronal receptor for BDNF (TrkB) appears to interrupt seizure propagation (Rivera et al. 2002; Ben-Ari et al. 2007, 2012; Kipnis et al. 2020). Moreover, the volume/ Cl^- -sensitive regulatory kinases of CCCs, known as WNK or AK/OSR1 pathways, could be useful in selective functional regulation of NKCC1 and KCC2 (Kahle and Staley 2009, Liu et al. 2020; Josiah et al. 2021; Lim et al. 2021). To conclude this section, it is important considering that NKCC1 and KCC2 also are involved in the pharmacoresistance in epilepsy (Sivakumaran and Maguire 2016; Löscher et al. 2020).

16.3.2 GABA_ARs Functional Expression in Pharmacoresistant Epilepsy

The potential involvement of the GABA system in the pathogenesis of drug-resistant human epilepsy has been mentioned in several studies and has provided information to consider it within the hypothesis of alterations in the pharmacological targets (Reviewed by Bazhanova et al. 2021). Most relevant data reveal alterations in numerous GABA_AR subunits found in biopsies from patients with epilepsy, including changes in extrasynaptic GABA_ARs (for review see Sperk et al. 2009), such as an increased expression of $\alpha 5$ subunit (that mediates tonic GABAergic inhibition in CA1 pyramidal neurons) and δ subunit (that mediates tonic GABAergic inhibition in DGCs) (Stell et al. 2003). These data are supported by evidence that tonic GABAergic inhibition is preserved in tissue from epileptic patients suggesting a significant role for extrasynaptic GABA_ARs in epilepsy. Similarly, using immunocytochemistry techniques, it has been shown alterations in distribution and location for GABA_AR subunits ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\beta 2$, $\beta 3$, and $\gamma 2$) in the resected hippocampus from mesial and nonmesial TLE patients compared with control tissues obtained from autopsies. Consistent with the severe neurodegeneration in the CA1 sector, significant decreases in $\alpha 1$ -, $\alpha 3$ -, $\beta 3$ -, and $\gamma 2$ -subunit immunoreactivity (IR) were detected in sclerotic but not in nonsclerotic specimens (Loup et al. 2000). In contrast, pronounced increases in the IR for all 3 β -subunits were observed in most parts of the hippocampal formation both in sclerotic and nonsclerotic specimens, being especially pronounced in the molecular layer of dentate gyrus and in the subiculum, where subunits $\alpha 3$ - and $\gamma 2$ -IR were also overexpressed. In situ hybridization experiments revealed mRNAs overexpression for $\beta 2$ and $\beta 3$ subunits in DGCs of patients with epilepsy with and without hippocampal sclerosis (Pirker et al. 2003). Data from our laboratory revealed increased mRNA expression of the $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 6$, $\beta 1$, $\beta 3$, $\gamma 1$, and $\gamma 2$ subunits in the cerebral cortex of patients with mesial TLE as well as overexpression of the $\alpha 1$, $\alpha 4$, and $\gamma 2$ subunits in the hippocampus (unpublished data). These results agree with those recently published by Castro-Torres et al. (2020), who showed that the GABA_AR $\alpha 2$, $\alpha 4$, $\alpha 5$, $\beta 3$, and $\gamma 1$ subunits, are significantly overexpressed in the cerebral cortex of pharmacoresistant TLE. It is important to consider that the altered GABA_A R composed of subunits $\alpha 4$ and $\alpha 5$ are involved in tonic inhibition (Farrant and Nusser 2005). These data indicate that seizure activity is associated with pronounced adaptive changes in the expression and assembly of GABA_AR subunits in pharmacoresistant TLE.

Likewise, different patterns of GABA_AR subunits expression have been observed in focal cortical dysplasia (FCDs), which involves a group of cortical development malformations frequently associated with drug-resistant epilepsy and are predominant among the pediatric population (Najm et al. 2022; Blumcke et al. 2017). FCDs are associated with architectural abnormalities and “immature” function of the neurons found in dysplastic areas (Cepeda et al. 2006). Indeed, abnormal synaptic transmission may be one of the key pathogenic factors leading to epilepsy in FCDs. In particular, the abnormalities in GABAergic neurotransmission play a key role in

this process due to GABA-mediated inhibitory neurotransmission becomes unable to regulate brain excitability and eventually drives epileptiform activity on its own (Hill and Bowery 1981; Sieghart 1995; Blauwblomme et al. 2019). Crino et al. (2001) described a decrease in the expression of the $\beta 1$ subunit in dysplastic neurons compared with pyramidal and heterotopic neurons. They also found a reduction in the expression of GABA_ARs $\alpha 1$, $\alpha 2$, and $\beta 2$ subunits in both dysplastic and heterotrophic neurons. Interestingly, in human TLE, most subunits expressed in the hippocampus seem to be upregulated (notably subunits $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1-3$, $\gamma 2$, and δ), indicating functional changes of GABA_ARs.

Currently, it was mentioned that the abnormal GABA chloride reversal potential, mainly determined by the deregulation of the expression of NKCC1 and KCC2, represents a significant factor in epilepsy that could lead to “depolarizing GABAergic transmission” (Kaila et al. 2014; Kahle et al. 2013; Ben-Ari 2012; Cherubini and Ben-Ari 2011). In addition, recent evidence supports the hypothesis that inflammatory stimuli, in particular IL-1 β can alter GABAergic neurotransmission by modulating the functional expression of the aforementioned CCCs (Pozzi et al. 2020). Alfano et al. 2022 indicated that IL-1Ra is also upregulated, suggesting an attempt to compensate for the IL-1 β increase in a cohort of FCDIIb samples. According to this information, endogenous anti-inflammatory cytokines fail to reduce neuroinflammation and can lead to brain hyperexcitability (Iori et al. 2016). The role of GABAergic transmission in this scenario is indeed pivotal. An interesting hypothesis revolves around the ability of aberrant GABAergic transmission to promote epileptogenesis in malformed FCD cerebral cortex, especially in pediatric cases (Cepeda et al. 2006; Cepeda et al. 2014). In fact, the shift in GABA reversal potential has been associated with a state of brain “dysmaturity” that is a hallmark of several neurodevelopmental diseases, such as tuberous sclerosis complexes (Talós et al. 2012; Ruffolo et al. 2016), Dravet (Ruffolo et al. 2018), and Rett syndromes (Ruffolo et al. 2019; Tang et al. 2016).

Additionally, other components of the GABA system have been studied in human epilepsy. For example, data obtained from samples of patients with TLE demonstrate that GABA transport is largely preserved (Mathern et al. 1999; Lee et al. 2006). Arellano et al. (2004) showed morphological and neurochemical reorganization of chandelier terminals and basket formations in the sclerotic hippocampus of patients with epilepsy. But these changes varied considerably across different hippocampal fields in each patient and among patients and were not correlated with the clinical characteristics or the degree of histopathological changes, such as granular cell dispersion, neuronal loss, and proliferation of mossy fibers (Arellano et al. 2004). Also, patients with epilepsy showed that certain surviving neurons adjacent to areas of neuronal loss were consistently innervated by dense basket formations in the subicular complex, whereas no apparent alterations were found in the cytoarchitecture or distribution of GABA transporter (GAT) type 1 (GAT-1). However, Schijns et al. (2015) reported that changes in the expression of GABA transporters correlate with the rate of hippocampal sclerosis. For example, the GAT-1 expression in hilus of patients with severe hippocampal sclerosis was approximately 7% lower

when compared with patients with mild hippocampal sclerosis or subjects without epilepsy. Similarly, the GAT-3 expression was approximately 5% lower in the severe hippocampal sclerosis group than in the mild hippocampal sclerosis or in the control group. In addition, samples of severe hippocampal sclerosis contained 34% fewer GAT-3-positive cells (not significant) than controls. Protein expression assessed by Western blot showed that GAT-1 was equally expressed in mild and severe hippocampal sclerosis samples, whereas GAT-3 was reduced by approximately 62% in severe hippocampal sclerosis samples. These data confirm that the expression of GABA transporters is spatially and specifically reduced in the brain of patients with epilepsy (Schijns et al. 2015). These results emphasize that GABAergic circuits present significant changes in human epileptic hippocampus that might be important in the pathophysiology of TLE associated with hippocampal sclerosis (Arellano et al. 2004). Similarly, preliminary data from our laboratory revealed a significant reduction in mRNA and protein expression of GAT-1 in the hippocampus of patients with TLE, a situation that could be associated with cell loss observed in the sclerotic hippocampus (Mathern et al. 2002).

GABA is the inhibitory neurotransmitter used by the majority of interneurons, and thus, the changes observed could represent compensatory plastic mechanisms to enhance the inhibition of some pyramidal cells. However, Cohen et al. (2002) showed that in the damaged subiculum, there is a subpopulation of pyramidal cells in which GABAergic effects result in depolarization instead of hyperpolarization. These cells present epileptiform activity and presumably act as pacemaker cells in generating interictal synchrony (Cohen et al. 2002). These results led to the possibility that GABAergic neurotransmission may have excitatory effects in the sclerotic hippocampus of patients with epilepsy. Additionally, data obtained from patients with pharmacoresistant epilepsy support that changes in the relative expression of NKCC1 (upregulation), and KCC2 (downregulation) may promote GABA excitatory effects and disrupt the neural inhibitory control facilitating to the pathogenesis of epilepsy (Muñoz et al. 2007; Liu et al. 2020).

16.4 Genetic Abnormalities in the GABAergic System Associated with Refractory Human Epilepsy

Genetic abnormalities are associated with complex diseases. Specifically, epilepsy is observed from the insertion or deletion of an entire chromosome or a single gene, also known as “copy number variants,” to abnormalities as simple as the change of a single nucleotide in a gene. These genetic alterations result in the pathological function of an encoded protein (Ritter and Holland 2020). In this case, we will discuss changes in molecules of the GABAergic system, such as the genes of the subunits that form the GABA_AR, GABA_BR, and gene SLC6A1, all related to different types of drug-resistant epilepsies.

16.4.1 Genetic Alterations of GABA_ARs Involved in Epilepsy

Mutations in GABA_AR genes can be divided into four general classes: (1) coding sequence missense mutations, (2) coding sequence nonsense mutations, (3) coding sequence frameshift mutations, and (4) noncoding sequence mutations (intronic or 5' upstream). GABA_AR mutations have been associated with changes in receptor function (impaired channel gating) and/or impaired receptor biogenesis (impaired subunit mRNA transcription or stability, subunit folding, stability, oligomerization, or receptor trafficking) (Macdonald et al. 2010). Figure 16.5 summarizes mutations in GABA_ARs related to epilepsy. The best known genetic alterations in GABA_ARs associated with human refractory epilepsy are described below.

Gamma-Aminobutyric Acid A Receptor- α 1 Gene or *GABRA1*, NCBI RefSeqGene NG_011548.1

This gene encodes the GABA_AR α 1 subunit, which is composed of 13 exons located on chromosomal region 5q34. Five reported variants of *GABRA1* differ in the 5'UTR. All have 1371 nucleotides, resulting in a protein of 456 amino acids (aa)

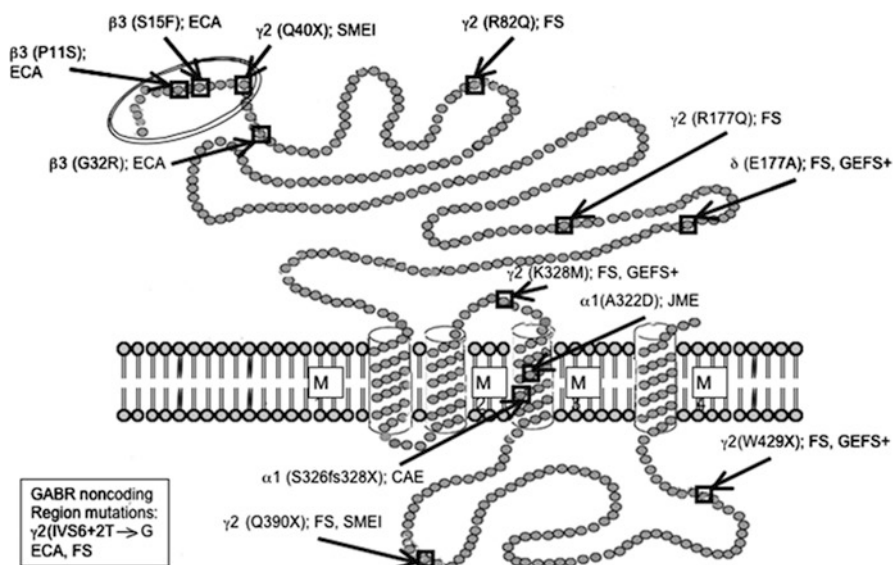


Fig. 16.5 The scheme shows GABA_ARs α 1, β 3, γ 2, and δ subunit mutations (small squares) associated with genetic epilepsy syndromes at their appropriate protein domain within the subunit. GABA_AR subunits are translated as a precursor protein whose signal sequence (double circle) is removed, leaving a mature protein consisting of a large extracellular domain at the N-terminus, four transmembrane domains (M1–M4), and a large cytoplasmic domain. (Modified from Macdonald et al. 2010)

(Kang et al. 1994). The relationship between *GABRA1* and susceptibility to juvenile myoclonic epilepsy (JME) was studied in four generations of a French Canadian family with an autosomal dominant pedigree pattern. The mutation screen showed a C to A (cytosine to adenine) substitution in this gene, resulting in a change in A322D (Ala to Asp in 322 aa) (Cossett et al. 2002). Subsequently, a study of 95 patients in this same population with idiopathic generalized epilepsy or IGE identified two families, one with the K353delins18X (25-bp insertion) mutation and the other with the A322D mutation (Lachance-Touchette et al. 2011). Both mutations cause misfolding of this protein, and therefore most of the protein is degraded. In this context, the residual nondegraded mutants reduce the surface expression of GABA_ARs by associating with wild-type subunits within the endoplasmic reticulum and preventing them from trafficking to the cell surface. In vitro cellular studies indicated that the A322D mutant reduces the surface expression of *GABRA3* by a more significant amount than $\alpha 1$ -containing receptors, thus contributing to cortical excitability. Therefore, these mutations allow a modest dominant-negative effect that likely contribute to the epilepsy phenotype (Ding et al. 2010).

In a German boy with childhood absence epilepsy (CAE), a de novo heterozygous 975delC mutation in the *GABRA1* gene was identified, resulting in a frame shift and premature truncation of the protein at codon 328 within the third transmembrane domain. The mutation was absent in parents and brothers and 292 ethnically matched controls (Maljevic et al. 2006).

In a cohort of 67 Australian and New Zealand patients with a clinical diagnosis of Dravet syndrome, also known as severe myoclonic epilepsy in infancy (SMEI), three different mutations were identified. Of these, only G251S showed a fivefold decrease (dose–response curves) in GABA sensitivity compared to WT in an in vitro model of *X laevis* oocytes (Carvill et al. 2014).

A 13-year-old boy from Austria was diagnosed at age of 18 months with cryptogenic focal epilepsy with complex focal seizures, severe developmental retardation, and optic atrophy. These alterations were associated with a heterozygous mutation in A332V of *GABRA1*. In vitro studies demonstrated that this genetic variant allowed normal basal protein levels and adequate receptor assembly with cellular localization to the cell surface. However, this variant affects the GABA function of receptor, driving a wide range of adaptive cellular responses (Steudle et al. 2020).

In a population of 671 patients from Japan with childhood epilepsy of different types, five missense mutations in *GABRA1* were identified in six unrelated patients with Othahara and/or West syndrome or early onset epileptic encephalopathy (EOEE): an R112Q mutation in the long extracellular N-terminus; P260L, M263T, and M263I in transmembrane spanning domain 1 (TM1); and p. V287L in TM2. Although functional alterations associated with these mutations were not demonstrated, the authors consider that their presence can cause epileptic encephalopathies (Kodera et al. 2016).

Gamma-Aminobutyric Acid A Receptor- α 2 Gene or *GABRA2*, NCBI RefSeq NG_012835.2

This gene encodes the GABA_AR α 2 subunit, composed of 14 exons located on chromosomal region 4p12. Sixteen reported variants of GABRA₂ differ in different regions of the gene. These variants range from 1104 to 1536 nucleotides, giving rise to a protein between 367 to 511 aa (Hadingham et al. 1993).

An 11-year-old girl with severe epilepsy and developmental delay was identified from a USA population of 279 individuals with epilepsy. The patient had a missense mutation in *GABRA2* (T292K), which reduced channel expression and produced mutant channels that were tonically open, even in the absence of GABA. These findings are supported by functional expression studies performed on human embryonic kidney 293T (HEK293T) cells (Butler et al. 2018).

A male infant, the first child of nonconsanguineous Ashkenazi Jewish parents with no family history of EOEE, and a de novo heterozygous mutation in N335H of *GABRA2* was identified. The variants' pathogenicity was predicted by in silico analysis (Orenstein et al. 2018).

In five children recruited from different hospitals with a diagnosis of epilepsy (EOEE, generalized tonic-clonic seizures, or autism spectrum disorder), four missense mutations located in other regions of *GABRA2* were identified: V284A, L291 V, M263T, and F325 L. Functional analysis demonstrated that four variants caused a loss of function, with the most severe impact seen for the L291V variant (Maljevic et al. 2019).

Gamma-Aminobutyric Acid A Receptor- α 5 Gene or *GABRA5*, NCBI RefSeq NG_032883.1

This gene encodes GABA_AR α 5 subunit, composed of 12 exons located on chromosomal region 15q12. Two reported variants of *GABRA5* that differ in the 5'UTR have 1.389 nucleotides to give rise to a protein of 462 aa (Wingrove et al. 1992).

A 2-year-old boy with severe epilepsy and developmental delay was identified from a group of 279 individuals with epilepsy from the USA. The patient had a missense mutation in *GABRA5* (V294 L). Functional expression studies on human embryonic kidney 293T (HEK293T) cells showed that the mutant subunit is incorporated into the channel with a ten-fold increased sensitivity to GABA compared to the wild type (Butler et al. 2018). Additionally in other study from China with 1969 patients with epilepsy and intellectual disability, two missense mutations in *GABRA5* were identified in two unrelated patients with EOEE: V294F and S413F. Functional expression studies were completed in rat primary hippocampal neurons and HEK293 cells. Both variants resulted in decreased GABA-evoked current amplitudes compared to the wild type, and mutant V294F protein reduced expression at dendritic GABAergic synapses compared to the wild type (Hernandez et al. 2019).

Gamma-Aminobutyric Acid A Receptor- β 2 Gene or *GABRB2*, NCBI RefSeq NG_047050.1

This gene encodes GABA_AR β 2 subunit, composed of 11 exons located on chromosomal region 15q34. Three reported variants of *GABRB2* are variable in the different areas of the gene. These gene variants range from 1.425 to 1.539 nucleotides, giving rise to a protein between 474 and 512 aa (McKinley et al. 1995).

In a group of 214 patients with seizure disorders, a 2-year-old boy with early myoclonic encephalopathy (EME) who had the heterozygous de novo 859A>C [T287P], missense mutation in the *GABRB2* gene was identified. Functional expression studies in HEK293 cells demonstrated a decrease in total and surface protein expression of the T287P mutant variant. GABARs containing the mutant beta-2 subunit were retained within the cell and associated with a significant dysfunction in the chloride channel (Ishii et al. 2017).

Gamma-Aminobutyric Acid A Receptor- β 3 Gene or *GABRB3*, NCBI RefSeq NG_047050.1

This gene encodes GABA_AR β 3 subunit, which is composed of 18 exons located on chromosomal region 15q12. Five reported variants of *GABRB3* are variable in the different areas of the gene. These gene variants range from 1.209 to 1.422 nucleotides, giving rise to a protein between 473 and 402 aa (Kirkness and Fraser 1993).

A group of 45 Austrian patients with diagnosis of CAE present 13 single-nucleotide polymorphisms (SNPs), of which 2 SNPs were novel, and four haplotypes between the promoter region and intron 3 were found. The association analysis using a Monte Carlo version of the multiallelic transmission disequilibrium test found that polymorphism -897C corresponding to SNP rs4906902 had the most critical association ($P = 0.007$). Reporter gene assays in the NT2 cell haplotype indicated that the haplotype -897C, -169T, and -66G promoter constructs conferred significantly lower transcriptional activity than the haplotype -897T, -169G, and -66C promoter constructs, which were overrepresented in the controls ($p < 0.0001$) (Urak et al. 2006).

Other polymorphisms related to CAE were found in members of two unrelated Mexican families with this disease. A heterozygous 31C>T transition in exon 1a of the *GABRB3* gene was identified, resulting in a P11S substitution in the alternative signal peptide. However, three unaffected family members from both families carried the mutation, indicating incomplete penetrance. This research group also identified a Honduran patient with CAE, a heterozygous 44C>T transition in exon 1a of the *GABRB3* gene, resulting in an S15F missense mutation; this genetic alteration was also present in his unaffected mother and half-brother. A third mutation, 962G>A transition in exon 2 of the *GABRB3* gene resulting in a G32R missense mutation was identified in two sisters of a Honduran family with EEG abnormalities (one sister has 2–4 Hz diffuse spike- and slow-wave complexes and febrile

convulsions; the other sister has 5–6 Hz frontocentral sharp waves). Mexican and Honduran patients were compared with 630 controls in which the mutations were not identified. In vitro cellular functional expression studies showed that P11S, S15F, and G32R mutants of the GABRB3 protein were hyperglycosylated and had reduced mean current densities compared to the wild type (Tanaka et al. 2008).

In patients with Lennox-Gastaut syndrome (LGS) and infantile spasms (IS), there are four de novo mutations in GABRB3. The missense mutations LGS-associated in GABRB3 (D120N, E180G, and Y302C) are located at β + subunit interfaces and reduce whole-cell currents by decreasing single channel open probability without loss of surface receptors. Apart from that, in IS patients, the N110D mutation in GABRB3 and the F246S mutation in GABRB1, both located at β -subunit interfaces, produced minor changes in whole-cell current peak amplitude but altered existing deactivation by decreasing or increasing single-channel burst duration, respectively. The missense mutations E180G (GABRB3) and F246S (GABRB1) also produced spontaneous channel openings of the GABA receptor (Janve et al. 2016).

Gamma-Aminobutyric Acid A Receptor- δ Gene or *GABRD*, NCBI RefSeq NG_008168.1

This gene encodes GABA_AR δ subunit and is located in chromosomal region 1p36.3. It comprises ten exons with 1359 nucleotides, resulting in a protein of 452 aa (Sommer et al. 1990).

In a small family from southern Australia, a heterozygous 659G>A (R220H) polymorphism in exon 6 of the GABRD gene in the N-terminal extracellular domain of the protein was identified. The polymorphism was found in 8.3% of patients with idiopathic generalized epilepsy (IGE), 3.1% with generalized epilepsy with febrile seizures plus (GEFS+), and 4.2% of control individuals (Dibbens et al. 2004). However, Lenzen et al. (2005) did not find an association between the R220H variant and idiopathic generalized epilepsy (IGE) or juvenile myoclonic epilepsy (JME) among 562 German patients and 664 controls (Lenzen et al. 2005).

Gamma-Aminobutyric Acid α Receptor- γ 2 or *GABRG2*, NCBI RefSeq NM_000806.5

This gene encodes GABA_AR γ -2 subunit located in chromosomal region 5q34 and shares approximately 40% sequence identity with the α and β subunits. It comprises 9 exons with 1371 nucleotides, resulting in a protein of 456 aa. There are three reported isoforms of GABRG2 (Wilcox et al. 1992).

In a four-generation family with a diagnosis of CAE and/or febrile seizures, a heterozygous G245A (R43Q) missense mutation in GABRG2 was found. This genetic alteration is associated with lack of sensitivity to diazepam. Three individuals with GEFS+ and two with myoclonic astatic epilepsy (MAE) were identified in the familiar lineage, confirming that the R43Q mutation contributes to GEFS +

syndrome. The authors suggest that even though both syndromes have different ages of seizure onset, and the physiology of absences and seizures are distinct, the R43Q mutation has age-dependent effects on various neuronal networks that influence the expression of these clinically particular but genetically related conditions (Wallace et al. 2001). **Functional in vitro expression studies** have demonstrated that the R43Q mutant briefly increases in temperature, resulting in impaired trafficking, accelerated endocytosis, and decreased surface expression, which could explain the triggering of seizures by fever in patients with this mutation (Kang et al. 2006).

In a screening of 1200 unrelated patients with various epilepsy phenotypes [GEFS+, febrile seizures, and idiopathic generalized epilepsy (IGE)], a bilinear family (obtained on 156 family members) initially described as “family G” was identified. In this family, a proband with a 1168C>T (Q351X) missense mutation in the GABRG2 gene associated with generalized epilepsy with febrile seizures plus type 3 (GEFS +3) was detected. Later, the proband developed Dravet syndrome (Harkin et al. 2002). Similarly, Kang et al. (2006) showed the same behavior for the Q351X mutant (Kang et al. 2006).

A family with CAE and febrile convulsions/seizures (FCS) was identified from 135 unrelated German patients with idiopathic absence epilepsy and compared with 154 unrelated and ethnically matched controls. In this family, the affected sister and father of the index patient but not the clinically unaffected mother carried the IVS6 + 2T-G mutation (thymine to guanine substitution occurring at the splice donor site of intron 6) of the GABRG2 gene. This genetic alteration suggests exon skipping, premature truncation, and a nonfunctional protein (Kananura et al. 2002).

In a family with febrile seizures, three affected members (two affected siblings and their father) were identified as carrying a heterozygous 529C>G transversion (R139G) found in the second benzodiazepine binding of GABRG2. The R139G mutation was absent in 368 control chromosomes. The paternal grandfather, reportedly unaffected, also carried the mutation, suggesting incomplete penetrance. All patients had normal mental development, and none developed epilepsy later in life. Functional in vitro expression studies showed that this mutation desensitized more rapidly the GABA_A receptor- γ 2 than the wild type and significantly decreased diazepam sensitivity (Audenaert et al. 2006).

16.4.2 Genetic Alterations in Gamma-Aminobutyric Acid B Receptor 2 or GABBR2, NCBI RefSeq NM_005458.7

This gene encodes subunit 2 of GABBR, composed of 22 exons located on chromosomal region 9q22.23, allowing a transcript of 2826 nucleotides to result in a protein of 941 aa (Kaupmann et al. 1998).

Two SNPs of *GABBR1* and four SNPs of *GABBR2*, all in intronic regions, were selected and genotyped in 318 patients with mesial TLE and 315 nonepileptic individuals in a Han Chinese population. SNPs rs29259 of *GABBR1* and rs1999501 and

rs944688 of *GABBR2* were thought to be associated with mTLE. After Bonferroni correction, these associations were not observed, and only the rs967932 A-allele of *GABBR2* was found to increase the risk of mesial TLE in the dominant model ($P = 0.036$). The frequency at which the haplotype G-C-A-C (rs3780428-rs1999501-rs967932-rs944688) of *GABBR2* occurred in mTLE patients was significantly higher than that in the controls (12.26% vs. 6.51%, $P = 0.0004$), and patients carrying this haplotype exhibited an earlier onset of mTLE ($P = 0.028$) (Wang et al. 2008).

An 18-year-old man with developmental and epileptic encephalopathy (DEE) was identified as a de novo heterozygous 2114T>A transversion (I705N) in the *GABBR2* gene (EuroEPINOMICS-RES Consortium 2014). Functional in vitro expression studies using HEK293 cells demonstrated reduced agonist-induced receptor activity (Yoo et al. 2017). An 1-year-old boy with DEE was identified as a de novo heterozygous c.2077G>T transversion (G693 W) in the *GABBR2* gene. The patient first had focal seizures at 11 months of age, followed by the onset of refractory epilepsy at 4.5 years of age. Without functional in vitro expression studies, the authors noted that *GABBR2* is involved in synaptic inhibition (Hamdan et al. 2017).

16.4.3 Genetic Alterations in Solute Carrier Family 6 Member 1 or *SLC6A1*, NCBI RefSeq NM_005458.7

This gene encodes a GABA transporter composed of 17 exons located on chromosomal region 3p25.3. Five reported variants of *SLC6A1* differ in different regions of the gene. These gene variants range from 1266 to 1800 nucleotides and give rise to a protein between 421 and 599 aa. (Lam et al. 1993).

A cohort of 569 individuals recruited from different hospitals with epileptic encephalopathy was a part of 85 patients diagnosed with MAEs, for which no molecular cause had been previously identified. In the MAE group, four children showed different missense mutations in the *SLC6A1* gene. The 131G>A (R44Q), 889G>A (G297R), 1000G>C (A344P), and 863C>T (A288 V) substitutions suggest a decrease in GABA transport activity or loss of GAT-1 function (Carvill et al. 2015).

16.5 GABAergic Agents as Treatment to Refractory Human Epilepsy

Drug resistance in clinical epilepsy is not fully understood, but several mechanisms have been proposed based on findings of modifications in the GABAergic system demonstrated in experimental models and in human tissue from patients with epilepsy supporting the “target hypothesis” (Kwan et al. 2011). In that work, molecular changes consistent with decreased drug sensitivity have been described. The

evidence from animal models suggests that lack of drug sensitivity associated with cellular/molecular/network changes are like those found in clinical populations.

Due to the multifactorial genesis of DRE and the difficulty in understanding how different mechanisms may interact with each other in the same patient or group of patients, the task of overcoming drug resistance with new drug treatments is still challenging. There are currently several drug candidates in clinical trials for the control of refractory epilepsy, many of them are modulators of the GABA_A receptor or GABAergic neurotransmission by mechanisms other than benzodiazepines such as cannabinoids.

Recent clinical trials indicate that cannabidiol (CBD) may have an effect in reducing the number of seizures in severe pediatric epilepsies such as Dravet syndrome, Lennox-Gastaut syndrome, and infantile spasms (Paolino et al. 2016; Brodie and Ben-Menachem 2018). The exact mechanism of antiseizure action of these cannabinoids is unclear. It is known that CBD can act as a positive allosteric modulator at all α -containing GABAA receptors, but with greater efficacy for the $\alpha 2$ -containing receptor. CBD and 2-AG (2-arachidonoylglycerol) showed greater efficacy at $\alpha 2\beta 2\gamma 2L$ and $\alpha 2\beta 3\gamma 2L$ receptors compared to $\alpha 2\beta 1\gamma 2L$ GABAA receptors, indicating a preference for $\beta 2/\beta 3$ receptors over $\beta 1$ receptors. It was suggested that the action of CBD on specifically configured GABAA receptors does not involve the classical benzodiazepine site and may have relevance to the anticonvulsant and anxiolytic effects of the compound. In addition, it was suggested that CBD could restore physiological GABAergic transmission (Bakas et al. 2017; Cifelli et al. 2020; Olafuyi et al. 2022).

The clinical trial of cannabidiol for drug-resistant seizures in Dravet syndrome was a double-blind, placebo-controlled study involving 120 children aged 2–18 years (NCT02091375). Patients were randomized to receive oral cannabidiol (20 mg/kg) or placebo in addition to standard antiepileptic treatment (Devinsky et al. 2019). Results showed that patients experienced a 50% reduction in seizure frequency compared to 27% with placebo, only 5% of patients were seizure free with cannabidiol. On the other hand, the trial of CBD treatment for Lennox-Gastaut syndrome was a double-blind, placebo-controlled trial involving 171 patients (NCT02224690). Patients were randomized to receive oral CBD (20 mg/kg) or placebo in addition to standard treatment with ASM (French et al. 2017). A change in seizure frequency from baseline and over the 14-week treatment period of 44% reduction was observed with treatment compared to 22% for the placebo group. Although the exact mechanism of CBD in these syndromes is unknown, it is likely that it acts in a way that is unique to certain GABA_A receptor modulating drugs, such as neurosteroids and benzodiazepines. Neurosteroids are known to preferentially bind to δ -type GABA_A receptors (Wu et al. 2013; Golub et al. 2023) and positive allosteric modulation at extrasynaptic GABA_A receptors contributes to tonic inhibition, thus promoting network shunting and reducing seizure susceptibility (Carver and Reddy 2013; Reddy et al. 2019). On the other hand, the anticonvulsant potential of CBD in combination with conventional GABAergic drugs and neurosteroids has not yet been investigated. Combination therapies of compounds with distinct GABAergic mechanisms may have a synergistic therapeutic efficacy in epilepsy (Löscher 2021).

Further studies are needed to better characterize CBD modulation of GABA_AR function and to identify the exact binding site of CBD to human GABA_ARs. However, CBD may be considered in the treatment of other neurological and psychiatric conditions in which GABAergic transmission is impaired.

Other proposed GABAergic agent is brexanolone (BXN) commonly known as allopregnanolone (AP). AP is a metabolite of progesterone and an endogenous inhibitory neurosteroid. Neurosteroids regulate neuronal excitability by potentiating synaptic and extrasynaptic GABA_A receptors. Therefore, although synaptic receptors are reduced during prolonged seizures, AP achieves its anticonvulsant activity by its activity at conserved extrasynaptic receptors, which mediate rapid inhibition in the brain. AP potentiation of GABA-evoked currents is well documented in the literature (Reddy 2010, 2011; Reddy and Rogawsky 2012; Kaminski et al. 2004; Rogawski et al. 2013).

A randomized, double-blind, placebo-controlled, phase I/II clinical trial to study the safety, efficacy, and tolerability of AP (SAGE-547) in patients with super-refractory *status epilepticus* (SE) (NCT02052739) was developed. Among the 25 patients who underwent AP treatment for 5 days, 19 patients were evaluable were successfully withdrawn from both AP and all other anesthetic agents (Kanes et al. 2016). Based on these results, the phase I/II trial supports further trials with AP in patients with SE and refractory epilepsy.

In other multicenter phase 1/2 study in patients with super-refractory status epilepticus, safety and tolerability of BXN were assessed, and the efficacy of BXN was also evaluated during and after withdrawal of an anesthetic anticonvulsant administered as a third-line agent. After 48 h of BXN infusion, anesthetic anticonvulsants were withdrawn while BXN was continued. In this pilot study, BXN was administered to 25 patients. In 22 patients, anesthetic anticonvulsants were gradually pointed, and in 16 of these patients (73%), seizure activity did not occur within 5 days of withdrawal of the anesthetic anticonvulsant (Rosenthal et al. 2017). These preliminary data indicated that allopregnanolone/brexanolone appears to be a promising compound in super-refractory status epilepticus.

However, in a randomized, double-blind, placebo-controlled phase 3 study, the primary endpoint, i.e., success in withdrawing third-line anesthetic agents and remaining free of SE was not achieved with BXN vs. placebo (43.9 vs. 42.4%; $p = 0.8775$) when added to standard therapy (SAGE therapeutics reports). In summary, the neurosteroid BXN was not shown to be effective in super-refractory SE after failure of GABAergic anesthetic anticonvulsants compared with placebo (Rosenthal et al. 2017).

Additionally, ganaxolone (GNX) is another positive allosteric modulator drug of synaptic and extrasynaptic GABA_A receptors with activity like that of AP and other neurosteroids of this class. The 3 β -methyl substituent in its chemical structure prevents its metabolism and oxidation at the 3 α -hydroxy moiety. Therefore, GNX does not have the hormonal activity and does not undergo back-conversion, avoiding the side effects, biotransformation, and tolerance associated with allopregnanolone (Turkmen et al. 2011). It has the same effects as endogenous AP, except that it does not activate the classical nuclear progesterone receptors. It is orally active and has

more favorable safety characteristics than AP. GNX may be suitable for the treatment of refractory epilepsy, as it does not appear to develop tolerance with chronic administration (Reddy and Woodward 2004).

GNX potentiates GABAergic tonic and phasic inhibition through activation of extrasynaptic and synaptic GABA_A receptors (Reddy et al. 2010, 2012). Phasic inhibition is fast and of short duration, whereas tonic inhibition persists for longer and sustained intervals. The modulatory activity of GNX is very similar to that of endogenous AP. However, synthetic analogs such as GNX were intentionally developed to avoid adverse effects by lacking affinity for steroid receptors in the brain.

The first randomized clinical trial (RCT) of GNX was conducted in patients with medically refractory complex partial seizures, prior to surgery. Fifty-two adult patients (age 18–65 years) were enrolled and randomized to GNX monotherapy or placebo for up to 8 days and with Lorazepam treatment (0.05 mg/kg) for the 48 hours to maintain seizures in an acceptable frequency range. GNX was administered according to the following escalation schedule: 1500 mg/day on day 1 and 1875 mg/day on the remaining days. Efficacy was assessed as the time from treatment with the full dose (day 2) to the occurrence of a seizure or other event leading to termination of study participation. Fifty percent of GNX-treated patients remained in the study through day 8, compared with 25% of patients in the placebo group (Laxer et al. 2000). However, the results of the analyses were not statistically significant, which could be attributed to the short duration of the study. An alternative analysis focusing on the difference in the number of patients in each treatment group who completed the trial according to protocol indicated a significant difference between treatments in favor of GNX. The tolerability of GNX was like placebo, with adverse events (AEs) primarily related to the central nervous system (CNS) and dizziness being the most common (Laxer et al. 2000). This study was the first “proof of concept” to clinically determine the anticonvulsant activity of GNX. However, it was limited by the small sample size and relatively short duration of treatment.

By other way, several clinical trials have recently been completed to test the efficacy and safety of oral GNX in patients with epilepsy. In a multicenter, Phase III, randomized, double-blind, placebo-controlled study of GNX as adjunctive treatment in adults with uncontrolled partial-onset seizures (NCT01963208), included 359 patients (179 on GNX and 180 on placebo). The results obtained revealed that GNX (1800 mg/day) induces a decrease of 21% in seizure frequency over 14 weeks versus 10.25% for placebo (Lappalainen et al. 2017). The study demonstrated safety and tolerability, and the most frequent adverse effects were somnolence, dizziness, and fatigue, which placed it as a safe drug.

Other drugs as GABAergic agents are two widely used diuretic drugs that have been implicated in the regulation of NKCC1 and KCC2: bumetanide, which blocks NKCC1 and furosemide, which acts on several types of ion transporters (Vanhatalo et al. 2009; Uwera et al. 2015). Furosemide acts on multiple proteins, such as KCC2, AE3, some GABA_AR subtypes, as well as carbonic anhydrase (Staley 2002; Maa et al. 2011). It exerts its anticonvulsant effect through a number of mechanisms regulation of [K⁺] and [Cl⁻] homeostasis, cell volume, and pH. Uwera et al. (2015)

studied the effects of furosemide on cesium (Cs)-induced epileptiform activity and found that it blocks the neuronal transporters KCC2 and AE3. Bumetanide regulates intracellular chloride ion concentrations of neurons (Ben-Ari 2012; Löscher et al. 2013; Puskarjov et al. 2014). Immature neurons are known to have high intracellular chloride, which drives GABA_A receptor-mediated chloride currents to be depolarizing and excitatory. By blocking NKCC in neurons, bumetanide decreases intracellular chloride concentrations. This concentration change makes the action of GABA_AR more hyperpolarizing. Furthermore, *in vitro*, and *in vivo* studies confirm that bumetanide can render immature GABA_A neurons from depolarizing to hyperpolarizing (Löscher et al. 2013). This driven inhibition is believed to have potential for the treatment of neonatal seizures, which often do not respond to traditional GABAergic treatment (Puskarjov et al. 2014).

Promising results have been obtained in rodent models, reaffirming that bumetanide prodrugs can cross the blood–brain barrier and can effectively target patients against disorders such as pharmacoresistant epilepsy (Erker et al. 2016). The first phase I/II clinical trials (NCT01434225) exploring the anticonvulsant efficacy of bumetanide (Jullien et al. 2016; Ben-Ari et al. 2016) was an exploratory dosing and pharmacokinetic study for the treatment of neonatal seizures in infants up to 44 weeks. The results revealed reduction of electrographic seizures by more than 80% during the third and fourth hour after administration of bumetanide. However, the study was terminated prematurely due to ototoxicity and lack of efficacy.

Additionally, Kahle et al. found that bumetanide does not induce antiseizure effects in the human neonate (Kahle et al. 2009). Indeed, the first phase I/II clinical trial with bumetanide in 14 neonates with seizures showed that this drug was not effective as adjuvant to phenobarbital and increased the risk of hearing loss (Pressler et al. 2015). Moreover, Eftekhari et al. (2013) were the first to demonstrate the efficacy of bumetanide on reduction of seizure frequency in three patients with TLE. Bumetanide decreased seizure frequency in all three patients and decreased epileptiform discharges in two of the three patients. The authors pointed out that the efficacy of bumetanide must be demonstrated in a larger number of patients for its use as an adjunctive treatment for drug-resistant TLE.

Other study was open-label, single-arm clinical trial was conducted to evaluate the safety and efficacy of bumetanide as an add on treatment in 30 patients with drug-resistant epilepsy and its relation to cation-chloride cotransporters NKCC1 and KCC2. The results showed that bumetanide is a safe and relatively tolerable drug with good antiepileptic properties, after 6 months of bumetanide add-on therapy, 70% of patients were responders and 18.5% became seizure free, and a reduced post-ictal period was reported by more than half of our patients. The therapeutic effects of bumetanide became visible 3 months after drug initiation when compared with baseline; 63% of patients had at least 50% fewer seizure attacks. By continuing treatment, 92.6% of the patients became responders during the second 3 months of study (Gharaylou et al. 2019). However, despite the results in these 2 clinical trials, the adverse side effects of bumetanide should be considered for its application in clinical practice. Therefore, it remains to be determined whether bumetanide will become a new drug against drug-resistant epilepsy.

16.6 Concluding Remarks

In accordance with all described above, modulation of GABAergic neurotransmission continues to be one of the main strategies in the treatment of epilepsy. Abnormalities in the GABA system, particularly at level of GABA_AR subunits, have been broadly associated with seizure generation, epilepsy, and pharmacoresistance, but whether these abnormalities are the cause or consequence of refractoriness remains to be elucidated. Changes in GABA_AR subunit assemblies, as in other multimeric receptors, lead to modifications in their sensitivity and responses to ligands, including the orthosteric agonist, which could reduce the efficacy of GABA in controlling seizure activity. In addition, clustering, trafficking, and recycling of the GABA_ARs can be altered by the subunits assemblies. The panorama can be more complicated if, in addition to GABA_AR, we consider other molecular components of the GABAergic system such as cation-chloride cotransporters as NKCC1 and KCC2, GABA transporters or GABA_BR, among others. However, considering all different epilepsy types and the relevant role of GABA in the neuronal excitability, as well as in the balance between hypersynchronization and desynchronization of neuronal activity, it is evident that modulation of GABA activity may improve seizure control even in pharmacoresistant epilepsy. Therefore, the GABA system must be further evaluated through experimental models, in epileptic tissue samples and in population genetic studies of patients with different types of epilepsy, to continue improving the treatment of this disease, particularly drug-resistant epilepsy.

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Chapter 17

Genes Involved in Pharmacoresistant Epilepsy



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Abstract This chapter is devoted to drug-resistant epilepsy and its genetic mechanisms. There are currently six hypotheses proposed for pharmacoresistant epilepsy. The genetic aspects of five of the six hypotheses are addressed in this chapter except from the intrinsic hypothesis as it does not comprise genetic mechanisms.

In the end, we propose two assertions in the definition of genetic “drug-resistant” epilepsies: (1) when the antiseizure medication (ASM) has a proven effect on the molecular lesion, but seizures persist in spite of ASM treatment; this latter, we believe is true genetic pharmacoresistant epilepsy and (2) epilepsies are drug resistant because the antiseizure drug ASM does not have an effect on the specific molecular lesion of the epilepsy syndrome. The epilepsy is supposedly “drug resistant,” but seizures do not stop because “the key does not fit the lock in the door”.

In diagnosis poor response to treatment, it is also important to consider that incorrect diagnosis in some epilepsies can also lead to “pseudo-drug resistance,” where mistreatment can lead to poor response or aggravation of seizures, this happens more frequently in genetic epilepsies.

Keywords Genes · Drug-resistant epilepsy · Epilepsy · Hypothesis, Pharmacogenomics · Genetic Epilepsies

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17.1 Genetics of Target Hypothesis

Target hypothesis consists in the lack of response or decreased sensitivity to the medication, as seizure control is not achieved, further changes occur in the structure and/or function of the ASM target, this leads to greater drug resistance in epilepsy (Juvale and Che Has 2021).

It is suggested that altered gene expression due to epigenetic regulation and/or the formation of receptor mosaics because of exogenous environmental factors such as the high frequency of seizures, may cause transcriptional or posttranscriptional changes with the consequent structural alterations that cause decrease effectiveness in various targets (Łukawski and Czuczwar 2021).

Despite target ineffectiveness, it is still crucial to use different therapeutic targets in patients with DRE, although it is still not possible to understand why people with drug resistance do not respond to the use of several drugs with different mechanisms of action that would try to compensate for these differences.

This theory involves voltage-gated ion channels and receptors of neurotransmitters and/or enzymes.

17.1.1 Genetic Variants of Voltage-Gated Ion Channels

Channels are essential in the generation and propagation of action potentials, therefore loss or gain-of-function mutations related to channel inactivation are relevant (Shlobin and Sander 2022). Although the main theme of the genetic variants of ion channels in epilepsy are channelopathies, there are other particularities to consider.

Voltage-Dependent Alterations of Sodium (Na⁺) Channels

These channels are the primary target of most ASMs, they act by blocking the resting phase (tonic block), selectively preventing the opening of the channel and the conductance of Na⁺. These channels are formed by an α subunit (pore-forming) and two β subunits (associated auxiliary), when modified they have a role in drug resistance, mainly in relation to the use of carbamazepine (CBZ) or phenytoin (PHT) (Łukawski and Czuczwar 2021).

The β subunit is responsible for modulating the membrane expression of the Na⁺ channel, its alterations cause incorrect protein folding, and/or abnormal expression of the channel, which are slowly inactivated, causing an increase in Na⁺ currents, thus way they promote a lower sensitivity to ASMs. The mutation in the gene that encodes the subunit has been related to genetic epilepsy with febrile seizures plus (GEFS+) (Juvale and Che Has 2021).

A relationship has been identified between the rs3812718 and rs2298771 variants of the *SCN1A* gene and the risk of resistance to CBZ, but the findings are

controversial. Given this, a meta-analysis was conducted and all controlled clinical trials on the association of *SCN1A*, rs3812718 and rs2298771 variants with CBZ resistance in epilepsy were included. A significant association was found between rs2298771 (GG vs. GA + AA) and CBZ resistance in patients of Asian origin with epilepsy, indicating that patients of Asian descent with epilepsy and *SCN1A* variant rs2298771, especially with the GG genotype, may be at risk of CBZ resistance. In addition, an interaction between the constitutive androstane receptor (CAR) variant rs2502815 and the CBZ response was observed, being identified for the first time a potentially significant interaction between the rs2502815 CAR variant and CBZ response in patients with epilepsy (Kong et al. 2021).

Voltage-Dependent Alterations of Calcium (Ca⁺) Channels

They are transmembrane channels formed by an $\alpha 1$ subunit that functions as the main pore and sensor during the change in potential. They are encoded by 10 genes and have several accessory subunits identified as β , γ , and $\alpha 2\delta$ (Juvalé and Che Has 2021; Sheilabi et al. 2020).

Ca⁺ channel has an excitatory function because it contributes to the initiation of the action potential by acting as a second messenger. There are 6 types (T, L, N, P / Q, R), which are subdivided according to the voltage required for their activation (high or low threshold). When these channels are altered, the activity and expression can be increased superficially in the membrane, causing a precipitous hyperpolarization, which leads to drug resistance due to the high frequency of seizures (Shin et al. 2008).

The T-type Ca⁺ channel is the only one that is activated with a low threshold. This channel is involved in the pathophysiology of absence-type seizures due to the generation of thalamocortical discharges. An imbalance between the $\alpha 1$ and $\alpha 1G$ subunits in Ca⁺ channel is likely to reduce the response to ethosuximide (ESM), lamotrigine (LTG), valproate (VPA), and zonisamide (ZNS), since the $\alpha 1G$ subunit has been linked to generation of spikes and waves of epileptiform discharge (Chioza et al. 2001).

17.1.2 Genetic Variants of Neurotransmitters Receptors

The functional brain requires optimal balance both excitatory and inhibitory inputs for efficient information processing at both the cellular and network level. At network level, this balance is provided by the organization of your main elements: the stimulation of glutamatergic basal cells and inhibition of GABAergic interneurons (Sears and Hewett 2021). A disturbance in balance has been implicated in the etiology and expression of epilepsy, as in the case of Dravet syndrome. However, not only pathogenic genetic variants are important in epilepsy but also nonpathogenic variants that influence response to treatment.

Alterations of Gamma Aminobutyric Acid (GABA) Channels

GABA is the main inhibitory neurotransmitter in the adult brain, but in the newborn, it behaves as an excitatory neurotransmitter. GABA_A receptors are assembled by 7 different subfamilies (α , β , γ , δ , π , θ , and ρ). The rearrangement of these subfamilies results in the absence of the specific ligand-binding site for benzodiazepines and barbiturates. Traumatic injury may also cause increased GABA_A inhibition causing neuronal synchrony or aberrant disinhibition of the epileptogenic network; both alterations lead to an increase in recurrent epileptic seizures and drug resistance (Juvale and Che Has 2021; Łukawski and Czuczwar 2021).

Alterations of the GABA_A receptor in animal models have been associated with neuronal loss of the hippocampus in the face of recurrent seizures and/or sustained *status epilepticus*. Theories hold that a transcriptional change occurs in the α subunit (reduction of the $\alpha 1$ subunit and increase of the $\alpha 4$ subunit) or change in the composition of subunits (loss of the $\gamma 2$ subunit and incorporation of the δ subunit), with activation of GABA_A receptors nonfunctional “leftovers” and an alteration in the phosphorylation state of this receptor (Juvale and Che Has 2021).

The influence of genetic variants on response to ASMs has proved to be controversial as the findings that described the G1465A variant of the GABA_{B1} receptor to be associated with susceptibility to develop temporal lobe epilepsy and to determine the severity of the epilepsy (Gambardella et al. 2003); while these results have not been supported by other authors (Ma et al. 2005).

Interestingly, the C588T variant of GABR_{G2} has been identified as a risk variant for the development of idiopathic generalized epilepsy in the Pakistani population, but this gene variant has not been identified to be associated with drug-resistant epilepsy (Saleem et al. 2022). Equally contrasting results were obtained in a study of three cohorts: mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) (prototype of ASM-resistant epilepsy syndrome), juvenile myoclonic epilepsy (prototype of ASM-responsive epilepsy syndrome), and non-epilepsy controls. The study population were ethnically matched South Indian ancestry and the results identified that rs211037 GABR_{G2} variant predisposes to epilepsy, irrespective of its phenotype, but not to ASM resistance (Balan et al. 2013).

Glutamate Channel Alterations

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). Its function is mediated through ionotropic glutamate receptors (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) [AMPA], N-methyl-d-aspartate [NMDA], and kainite, as well as metabotropic receptors (Juvale and Che Has 2021).

NMDA receptors have magnesium ions blocking their pores, AMPA receptors are easily activated, generating rapid excitatory neurotransmission. The expression, composition, and location of both receptors depend on the amount of presynaptic

glutamate released and the strength of the resulting synapses, thus generating long-term potentiation and/or depression (Juvale and Che Has 2021).

During seizures, there is abnormal excitation in the brain, resulting in an alteration in AMPA receptor expression, which results in recurrent seizures and neural circuit remodeling. The same can happen with NMDA receptors but with slower activation, but once activated there is a constant influx of Ca^{+} into the neuron and with them a greater long-term potentiation arises that turns out to be even more damaging (Juvale and Che Has 2021).

Both receptors in a patient with poorly controlled epilepsy contribute to an imbalance and overload the inhibitory system. Moreover, various studies have observed a more specific role after NMDA activation because the internalization of GABA receptors is mediated through clathrin, not only reducing inhibition but also avoiding reaching the target.

The genes encoding the glutamate receptor *Gríá2* and *GluR2* have been found altered in epileptic tissue by epigenetic regulation regarding both histone acetylation and DNA methylation. Glutamine synthetase (GS) responsible for glutamate synthesis is downregulated in hippocampal astrocytes, generating astrogliosis and with them deregulation of glutamate homeostasis, which has also been related to susceptibility to febrile seizures in animal models (Hauser et al. 2018).

17.2 Genetics of Transporter Hypothesis in Drug-Resistant Epilepsy (See Fig. 17.1)

The transporter hypothesis is one of the two most inquired hypotheses regarding DRE. Resistance may be evident from the onset of ASM therapy or after an adequate initial response. There may even be cross-resistance to several ASM as a result of overexpression of transport proteins of membrane (Leandro et al. 2019). Various ASMs, their mechanisms of action, and their corresponding transporters are shown in Table 17.1.

According to this theory, excessive activation of P-glycoprotein (P-gp) leads to resistance of ASMs due to excessive clearance of these compounds through the blood–brain barrier back into the blood, resulting in scarce penetration of these drugs in the brain (Miller et al. 2008; Rizzi et al. 2002; van Vliet et al. 2007) (Fig. 17.1). There is evidence of P-gp overexpression in the brain induced by prolonged seizures, which may explain why delaying the start of treatment leads to a decrease in the effectiveness of ASMs (Sisodiya and Thom 2003).

Probable mechanisms associated with the rise in output transporters are inflammation and oxidative stress, drug-mediated induction, the release of glutamate during seizures, and the presence of genetic polymorphisms (Leandro et al. 2019). In this section, we will address the influence of genetic variants on the expression of these transporters.

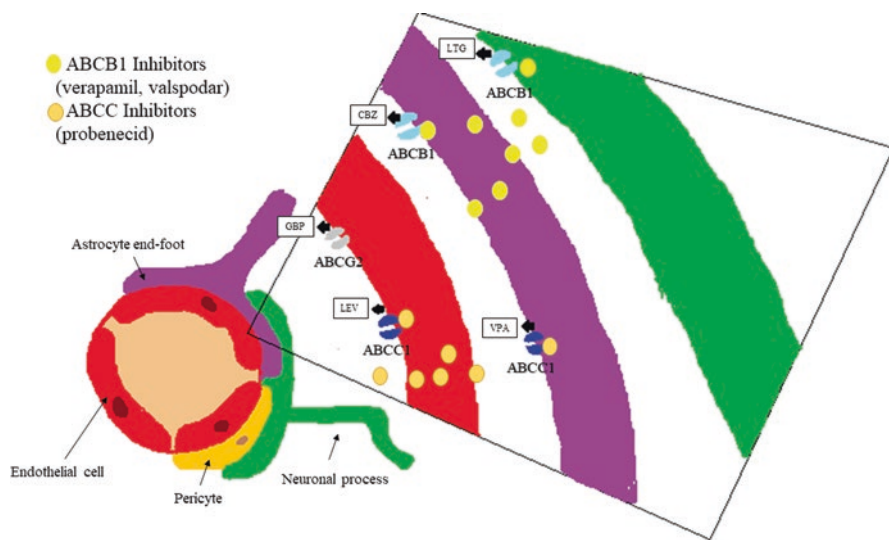


Fig. 17.1 ABC transporters in drug-resistant epilepsy. (a) Neurovascular unit made up of endothelial cells (red), pericyte (yellow), astrocytes (purple), and neurons (green). (b) ABC transporters in blood–brain barrier (BBB): ABCB1 transporter is upregulated in neurons and astrocytes (sky blue) of epileptogenic tissue and some antiseizure drugs affected by the overexpression of this transporter are phenytoin (PHT), phenobarbital (PB), lamotrigine (LTG), and carbamazepine (CBZ); ABCG2 transporters are overexpressed in astrocytes and/or dysplastic neurons (blue) of epileptogenic tissues and suggested substrates are phenytoin (PHT), phenobarbital (PB), levetiracetam (LEV), carbamazepine (CBZ), and valproate (VPA); finally, ABCG1 transporter is expressed in the endothelial cells of the BBB (gray) and suggested substrates are phenobarbital (PB), levetiracetam (LEV), tiagabine (TGB), and gabapentin (GBP)

17.2.1 The ABC Transporters (ATP Binding Cassette)

The *ABCB1* gene, encoding P-gp is located on chromosome 7 (7q21.1), contains 28 exons and is highly polymorphic. Variant genetic of this gene may be related to the effectiveness of antiseizure therapy and a rise in its expression leads to an increase in the amount of P-gp in the blood–brain barrier and astrocytes (Smolarz et al. 2021). Some genetic variants identified in *ABCB1* are in the coding part of the gene, and those located in exon 26 seem to have functional significance. There are three single nucleotide variants whose allele frequency appear to be of biological significance and are being intensively studied in the context of drug-resistant epilepsy: in exon 12 (rs1128503, 1236C>T), in exon 21 (rs2032582, 2677G >T/A) and in exon 26 (rs1045642, 3435C>T) (Skalski et al. 2011).

It has been found that the genotype C3435T/CC is associated with an increase in P-gp expression, which affects the concentration of ASMs in plasma of patients with DRE epilepsy (Lazarowski and Czornyj 2011; van Vliet et al. 2010). Pharmacogenomic studies regarding membrane drug transporters have found the association of DRE with the genotype 3435CC of the *ABCB1* gene (Siddiqui et al.

Table 17.1 Antiseizure medications, their mechanisms of action, and their corresponding transporters

Antiseizure Medication	Mechanism of action				Transporter						
	Na ⁺ Channel Blockade	T-type Ca ²⁺ Channel Blockade	Ca ²⁺ Channels Blockade	K ⁺ Channel Blockade	GABA Agonist	Glutamine Antagonist	SV2A Action	P-gp	MRP	MDR	Target
Carbamazepine	+	–	+	–	+	+	–	+	+	+	+
Clobazam/Clonazepam	+	–	+	–	+	–	–	?	?	–	+
Ethosuximide	–	+	–	–	–	–	–	–	?	?	?
Felbatame	+	–	+	–	+	+	–	+	–	+	?
Gabapentin	+	–	+	+	+	+	–	+	?	+	?
Lamotrigine	+	–	+	+	–	+	–	+	–	+	–
Levetiracetam	–	–	+	+	+	–	+	–	–	–	?
Oxcarbazepine	+	–	+	+	–	+	–	+	?	?	?
Phenobarbital/Primidone	+	+	+	–	+	+	–	+	–	–	+
Phenytoin	+	–	+	–	+	–	–	+	+	+	+
Pregabalin	–	–	+	–	–	+	–	?	?	?	?
Topiramate	+	–	+	+	+	+	–	+	?	+	?
Valproate	+	+	+	–	+	+	–	?	+	+	–

+ = effect
– = no effect
? = unknown

2003; Soranzo et al. 2005). However, studies of the relationship between *ABCB1* genetic variants and response to antiseizure treatment have yielded contradictory results (Cascorbi et al. 2001; Mosyagin et al. 2008; Sills et al. 2005; Tan et al. 2004; Vahab et al. 2009; von Stülpnagel et al. 2009).

Because there are differences in the efficacy of ASMs, studies have been conducted to assess the impact of genetic variants of membrane drug transporters on specific drugs. The effect of variants in the gene encoding P-gp on levetiracetam (LEV) disposition in children with epilepsy have been described (Zhao et al. 2020). It is reported an association to specific variants that may affect LEV therapeutic efficacy in children with epilepsy. Also, *ABCB1* gene loci genotypes have been compared in children with epilepsy and healthy children, identifying genotypes that are associated with DRE (Gao et al. 2019).

On the other hand, a strong association between genetic variants rs2235048, rs1045642, rs2032582, and rs1128503 of the *ABCB1* gene with ASM resistance has been demonstrated among women, but not men. This data suggests that there is a gender-dependent relationship between *ABCB1* genetic variants and the response to ASMs (Tamimi et al. 2021). However, more research is needed to confirm this association.

Furthermore, there is a dilemma about the influence of the genetic variants of the *ABCC2* and *ABCG2* transporters in altering the response to ASMs, with controversial and inconclusive results (Al-Eitan et al. 2019; Chen et al. 2019; Shen et al. 2016; Yang et al. 2019). A meta-analysis identified a significant association of the variant *ABCC2* rs717620 with ASM resistance in the general population, as well as an association of *ABCC2* rs3740066 with resistance to ASM. *ABCG2* rs2273697, *ABCG2* rs2231137, and rs2231142 were not associated with response to ASMs in the meta-analysis (Zan et al. 2021).

Therefore, therapies focused on combating drug resistance through direct inhibition of P-gp efflux transporters have been created. There are four generations of inhibitor drugs to be mentioned: (1) first generation, nonspecific for P-gp: cyclosporine A and verapamil; (2) second generation, more specific for P-gp but interfere with the cytochrome CYP3A4 metabolizing enzyme: valsopodar (cyclosporin A analog); (3) third generation, specific for P-gp and do not interfere with enzymes that metabolize drugs: Tariquidar; (4) fourth generation, still under study for its use in humans: cyclic peptide QZ59SE and the natural compounds lamellarin and gomisin (Tang et al. 2017). However, the use of specific P-gp inhibitors is not without concern, as systemic P-gp inhibition could increase plasma concentrations of drugs and toxins, potentially leading to systemic toxicity (Czornyj et al. 2022; Tang et al. 2017).

Among the P-gp inhibitors, the most used in epilepsy research is verapamil as adjuvant therapy in DRE. Its use is limited by its side effects on heart rate, blood pressure, skin rashes, and foot edema. Despite the fact that in an open non-placebo controlled study of 19 patients, in which verapamil was used without reports of effects on hemodynamics and a decrease of seizures by 50%, it is necessary to have more solid evidence with double-blind studies with larger populations. Nifedipine and diltiazem have also been coadministered with ASMs to inhibit P-gp, increasing brain plasma levels of ASMs, achieving better control of seizures. Moreover, as

medications that inhibit Ca^{+} channels, they could have an intrinsic activity and their inhibitory effect on CYP3A4 is difficult to differentiate from the effect of P-gp inhibition (Leandro et al. 2019; Tang et al. 2017).

17.3 Genetics of Neural Networks Hypothesis

The basis of this hypothesis is that recurrent seizures cause cell death, and the remodeling of these circuits can result in aberrant excitatory networks, either they have hyper excitatory properties or they do not respond to endogenous inhibitory mechanisms or to the action of the ASMs by not allowing them to reach their targets. It is suggested that, under the command of an erroneous message generated by recurrent seizures, the damaged brain tissue will spread to nonphysiological regions, forming abnormal connections with neurons far from their original synapses, all this through neuronal degeneration, necrosis, gliosis, sprouting, synaptic reorganization, and finally network remodeling (Fang et al. 2011).

Before the emergence of the neural network theory, it was already suggested that hippocampal sclerosis might play a causal role in the mechanisms of drug resistance in MTLE, and how its resection allowed seizure freedom in almost 60% of patients, although later it was corroborated that not all postoperative patients became drug sensitive. The experimental models carried out by Volk et al. and Bethmann et al. compared hippocampal damage in drug-sensitive epileptic rats with those that were drug-resistant. More than 90% of drug-resistant animals showed significant neuronal loss in CA1, CA3c/CA4 and in the dentate gyrus (Löscher et al. 2020). These structural changes could be the basis of the affection in multiple functional systems that patients with DRE suffer over time and evolution of the disease; for example, in executive functions, language and memory (Lazarowski et al. 1999).

The molecular basis of the network hypothesis can involve changes in multiple genes related to the cytoskeleton (Rho-family GTPase: Cdc42, RhoA, N-WASP, and actin related protein 2/3), neuronal plasticity, and structural reorganization identified in patients with DRE and mostly associated with the axonal growth cone. These findings expose how epileptogenesis shares characteristics with normal neurodevelopment (Fang et al. 2011). The greatest weaknesses of this hypothesis are basically the impossibility to find, to date, an adequate animal model for its reproducibility and the fact that these brain morphological changes do not always lead to DRE.

17.4 Gene Variant Hypothesis

This theory postulates that there are variations in genes that encode enzymes involved in the metabolism of ASMs, ion channels or neurotransmitter receptors that are the target of these drugs. These changes cause alterations in the

pharmacokinetics and pharmacodynamics of ASMs and compromise their efficacy (Łukawski and Czuczwar 2021; Pérez-Pérez et al. 2021). For example, a strong association was found between low activity of CYP2C9 alleles (CYP2C9*2 and CYP2C9*3) and a decrease in the required dose of phenytoin. An intronic single nucleotide polymorphism found in the *SCN1A* gene (IVS5-91G→A or rs3812718) was associated with higher required doses of CBZ and PHT. There is a high frequency of the *SCN1A* IVS5-91 AA genotype in patients with CBZ resistance. Many other single nucleotide polymorphisms associated with Na⁺ channel genes are currently under study (including *SCN1A* c.3184 A→G and *SCN2A* c.56 G→A).

The genetic associations require subsequent confirmation in larger populations, which is difficult because they are infrequent alleles, so the impact of this theory alone remains very limited.

It is worth mentioning the epigenetic changes that are not included in this or other DRE theories. Whether through DNA methylation, posttranslational modifications in histones, or noncoding RNAs, recurrent seizures can produce epigenetic changes, which could modify the response to drugs. On the other hand, it is believed that the ASMs by themselves could induce epigenetic changes that favor drug resistance. There is still no experimental evidence for these (Tang et al. 2017).

17.5 Genetics of Pharmacokinetic Hypothesis

Several patients with DRE had serum concentrations below the range considered therapeutic despite adequate adherence (Lancelin et al. 2007; Lazarowski et al. 1999; Mohammed Ebid et al. 2007). This hypothesis should be understood more specifically as the abnormalities in the metabolism and elimination of ASMs (Pérez-Pérez et al. 2021). It indicated that DRE can be explained by the low availability in the amount of ASM that enters the brain to reach the epileptogenic focus, since there is overexpression of ASM efflux transporters in other organs: intestine, liver, and kidneys (Tang et al. 2017).

Up to 90% of all prescribed medications are metabolized by CYP450 (CYP) enzymes. CYP3A4 represents more than 30% of hepatic CYPs, but there are others that are also of great importance for drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP2D6. Indeed, they are also expressed in endothelial cells and in the blood–brain barrier (Ghosh et al. 2010, 2011). They are encoded in genes that are highly polymorphic, such as *CYP2D6*, with more than 100 known allelic variants. Genetic variants can modify the function of the P450 enzymes (Feng et al. 2018). In fact, large ethnic and intersubject variations of these enzymes can explain partial or complete loss of enzyme function.

Regarding PHT, 13 alleles of the CYP2C9 gene have been identified, of which CYP2C9*2 and CYP2C9*3 resulted from mutations in the CYP2C9*1 coding sequence leading to the substitution of one amino acid—R144C and I359L, respectively. This is associated with reduced activity of the enzyme to metabolize PHT

(Brandolese et al. 2001). Other study shows that patients carrying at least one mutant CYP2C9 allele required 37% lower doses of PHT than the wild-type subjects (van der Weide et al. 2001). Another study in children demonstrated an important role of the allelic variant CYP 3A4*1B as a risk factor for the development of drug resistance (López-García et al. 2017). Moreover, it has been identified that the genetic variants rs1799853 and rs1057910 CYP2C9 and the polymorphism rs4244285 CYP2C19 may be associated with the occurrence of DRE in children (Makowska et al. 2020).

17.6 Pharmacogenetics of DRE in Children

DRE occurs frequently in epileptic syndromes such as Dravet syndrome during infancy (Gonzalez-Giraldo and Sullivan 2020), thus pharmacogenetic research in this age group has been increasing. A systematic review identified the genetic variants associated to the efficacy of ASMs in children with DRE. In this review included case-control and cross-sectional studies with analysis of ion channel genes, genes coding for drug transporters, and enzymes genes. Summarizing the results, variants of the ABCB1 gene have been associated with high-risk of resistance to ASMs, as S893A polymorphism (T allele, Ser893Ala) that was associated with risk of resistance to LTG in absence epilepsy, and C and T alleles of C3435T polymorphism that were shown to predispose to more resistant disease (Gogou and Pavlou 2019). In addition, while the variants of SCN1A gene and cytochrome P450 gene had a significant impact (either positive or negative) on responsiveness to ASMs, variants of uridine diphosphate glucuronosyltransferase UGT2B7 gene were associated with increased remission rate in children with generalized seizures (Gogou and Pavlou 2019). This demonstrates the impact of genetics on the development and management of DRE in children.

17.7 Genetic Epilepsies “Difficult to Treat”

Pathogenic alterations or mutation in genes and structural abnormalities in chromosomes (deletions, insertions) are responsible of a variety of epilepsies. In some, the possibility of DRE is high. Several mechanisms may be interrelated between genetic disorders and the presence of epilepsy. It is important to distinguish between the susceptibility to generate epilepsy caused by a functional abnormality of a gene and epilepsy that results from structural or functional abnormalities in a chromosome. Some genetic disorders relate more to certain types of epilepsy, but overall, any seizure type may be present. Table 17.2 summarizes some genetic pharmacoresistant epilepsies.

Table 17.2 Genetic pharmacoresistant epilepsies

	Disease	Phenotype	Genotype	References
Genes involved in drug resistant epilepsy	Dravet Syndrome	Normal development before onset Seizures initially induced by fever. Begin during first year of life Ataxia, mental decline EEG normal initially, generalized spike-wave activity	<i>SCN1A</i> <i>SCN9A</i> <i>GABRG2</i>	Dravet (1978), Ohmori et al. (2002), Singh et al. (2001), Harkin et al. (2007)
	Lafora disease	Insidious onset of progressive neurodegeneration, myoclonic jerks, generalized seizures, and often visual hallucination. Progressive cognitive decline resulting in dementia. Lafora bodies	<i>NHLRC1</i> <i>EMP2A</i>	Chan et al. (2003), Ganesh et al. (2002a, b)
	Unverricht-Lundborg disease	Begin between 6 and 13 years of age. Stimulus-sensitive myoclonic jerks, ataxia, incoordination, tremor, dysarthria. The disease stabilizes in early adulthood. No cognitive decline	<i>CSTB</i>	Shahwan et al. (2005)
	Pharmacoresistant MTLE with HS ^a	Febrile seizures, complex focal seizures. Epigastric aura, fear and orolimentary automatisms	<i>ABCB1</i>	Kubota et al. (2006)
Genetic alterations associated with drug resistant epilepsies	GLUT1 deficiency syndrome	Infantile-onset epileptic encephalopathy, microcephaly, incoordination and spasticity. Hypoglycorrhachia (<40 mg/dl) and low lactate	<i>SLC2A1</i>	Klepper and Voit (2002)
	Tuberous sclerosis	Hamartomas in multiple organ systems, epilepsy, autism, behavioral problems, angiomyolipomas, pulmonary lymphangioleiomyomatosis, melanotic macules, facial angiofibromas	<i>TSC1</i> <i>TSC2</i>	Povey et al. (1994)

(continued)

Table 17.2 (continued)

	Disease	Phenotype	Genotype	References
Chromosomal alterations associated with drug resistant epilepsies	Ring chromosome 20 Syndrome	Behavioral restlessness and aggression Mental retardation appearing after apparently normal development in a child without dysmorphic features Atypical absence status with diffuse 2–3 Hz slow waves and spikes of possible mesial frontal origin	Ring20	Canevini et al. (1998)
	Fragile X	Learning disorders, hyperactive, autistic child delayed speech, hypotonic, hyperelastic joints, macroorchidism, narrow, long face, large ears with small mandibles and focal epilepsy.	Xq27.3	Fu et al. (1991)
	Miller Dieker	Lissencephaly microcephaly, facial dysmorphism, cardiac malformations, growth retardation, mental deficiency, hypotonia, and intractable seizures. EEG: focal or multifocal spike-wave discharges, bisynchronous bursts of diffuse paroxysmal activity, and high-voltage diffuse rhythmic theta and beta activity	17p13.3 Deletion or mutation in <i>LIS 1 gene</i>	Miller (1963), Dobyns et al. (1993)
	Angelman Syndrome	Mental retardation, microcephaly, movement disorder, abnormal behaviors, and severe alterations in language. Myoclonic jerks, focal and generalized status epilepticus	Maternal deletion on 15q11-q13	Angelman (1965), Minassian et al. (1998)
	Prader Willi Syndrome	Hypotonia, obesity, mental retardation, short stature, hypogonadotropic hypogonadism, and seizures	Paternal deletion <i>SNRPN gene</i>	Cassidy et al. (1984)

^aMesial temporal lobe epilepsy with hippocampal sclerosis

17.8 Conclusions

17.8.1 Limitations of the Gene Hypothesis

To prove a given drug resistance theory, it is important to show that the subgroup of patients with DRE has differences in their receptors in comparison to those of responders. However, this is difficult to achieve because patients who respond to

ASMs are not subjected to epilepsy surgery or further study, although, sometimes as controls.

Any proposed mechanism for drug resistance must meet the following requirements to be considered valid: be detectable in epileptic brain tissue, have a pathophysiological mechanism demonstrable in human epilepsy, and, when modified, must affect the phenomenon of drug resistance (Sisodiya and Thom 2003).

The fact that most patients are resistant to multiple treatments, including several ASMs with different mechanisms of action, suggests that other less specific or unknown mechanisms with some commonality about ASM cellular or network actions contribute to drug resistance, or that more than one mechanism may be involved (Löscher et al. 2006). Some changes were induced only transiently in animal models of epilepsy which do not necessarily explain chronic pharmacoresistance (Deeken and Löscher 2007; van Vliet et al. 2005). According to Schmidt and Löscher (2009), the intrinsic hypothesis lacks studies and “a subgroup of patients with a higher seizure frequency at the onset of treatment will become seizure-free but require higher serum concentrations of ASMs to do so than those with a lower seizure frequency, and this hypothesis does not have a genetic component.”

17.8.2 How to Define Genetic Drug-Resistant Epilepsies?

This chapter leads us to the question—what is the definition of genetic “drug-resistant” epilepsies? In the end, we propose two assertions: (1) epilepsies are drug-resistant because the ASM does not have effect on the specific molecular lesion of the epilepsy syndrome. The epilepsy is supposedly “drug resistant” and seizures do not stop because “the key does not fit the lock in the door” and (2) when the ASM has a proven effect on the molecular lesion, but seizures persist despite a proper treatment; this later, we believe is true genetic pharmacoresistant epilepsy. Therefore, we favor the “intrinsic disease” mechanisms as an explanation for the resistance in genetic epilepsies (Fig. 17.2).

17.8.3 Future Directions

The question is how to tackle the problem in patients. A recommended step now is to genotype epilepsies and use the information obtained to guide ASM administration. It would be ideal to perform a whole or large scale pharmacogenomic study of how ASMs effects are genetically determined to look for responders and nonresponders and identify patients that could develop side effects as consequence of specific ASMs.

A large-scale pharmacogenomic study could also be done in patients who were submitted to surgery for DRE. Surgical specimens could be reviewed for their neuropathologic and biochemical abnormalities and correlate the findings with whole genome sequencing.

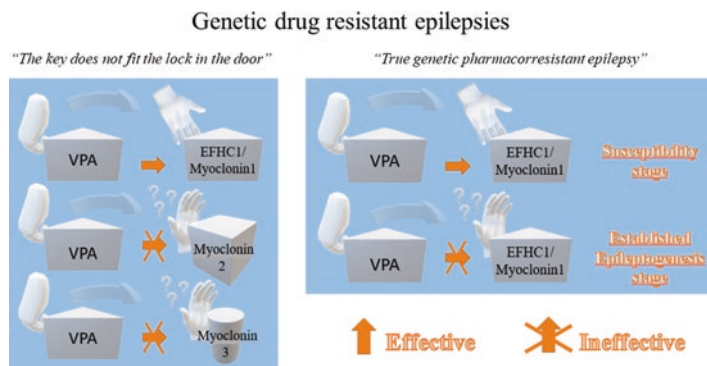


Fig. 17.2 (a) *EFHC1* /Myoclonin mutations in juvenile myoclonic epilepsy (JME) frequently respond to treatment with valproate (VPA); however, other JME mutations (illustrated as Myoclonin 2 and Myoclonin 3) might not achieve seizure control due to a different molecular lesion unresponsive to VPA therapy. (b) *EFHC1* /Myoclonin mutations in a susceptibility or early stage respond adequately to VPA treatment, whereas in established epileptogenesis drug resistance is seen even though the same molecular lesion is present; therefore, a true genetic pharmacoresistance is encountered

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Chapter 18

Drug-Resistant Epilepsy and the Influence of Age, Gender, and Comorbid Disorders



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Abstract In the study of drug-resistant epilepsy (DRE), certain important characteristics remain poorly understood such as the age and gender of patients and the presence of comorbidities. Several studies have revealed a higher risk of DRE when seizures occur during the neonatal period compared to the onset of epilepsy later in life. However, most of clinical studies are based on data from heterogeneous groups in terms of the age of the patients, creating difficulties for the adequate establishment of risk factors and prevention strategies that contribute to the prediction of DRE. The presence of comorbidities in patients with DRE negatively impacts the quality of life. This association may be due to the existence of common pathogenic mechanisms including endocrine disorders, neuroinflammation, disturbances of neurotransmitters, and stress. Most recent studies propose a role of adenosine deficiency and localization of epileptic foci in the development of comorbidities in DRE.

Further research of these factors is needed to design novel effective and specific therapies.

Keywords Drug-resistant epilepsy · Age · Gender · Comorbidities

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18.1 Introduction

Epilepsy is a devastating condition that affects approximately 46 million people globally (Beghi 2020; GBDE 2019). Around one-third of patients with epilepsy have drug-resistant epilepsy (DRE), in other words, they unsuccessfully respond to pharmacological treatment (Dalic and Cook 2016; Kwan and Brodie 2000; Picot et al. 2008). This multidimensional disorder involves several factors that directly affect the prognosis and effectiveness of the treatments. An important feature of most basic and clinical studies of DRE is that they usually do not consider factors such as age, gender, and the presence of comorbid disorders. For example, it has been shown that young and aged subjects are more vulnerable populations to epileptic activity (Brodie et al. 2009), and elderly patients with DRE show a cognitive deterioration (Sarkis et al. 2018). Regarding the influence of gender on the development of epilepsy has been suggested in numerous investigations. Some studies have revealed that females are more resistant to the development of seizures. However, at this time there is no convincing evidence to establish the role of gender in the development of DRE. Recent studies determined that gender is not a risk factor for the development of DRE (Kalilani et al. 2018). In addition, patients with DRE frequently display additional neuropsychiatric disorders (Kanner 2012, 2016). Remarkably, in some patients, it has been demonstrated that the impact of the comorbidities is even more severe than the epilepsy itself (Kanner 2016). This chapter will explore the influence of the age, gender of the patients, and the presence of comorbid disorders on the development of DRE.

18.2 Role of Age on DRE

The prevalence of DRE is bimodal with a childhood peak and a late surge in the elderly (Bartolini et al. 2022). It has been shown that 7–25% of children can develop DRE (Sultana et al. 2021; Berg et al. 2012). Meanwhile, 15–40% of adult patients are refractory to pharmacological treatment (Denton et al. 2021; Sultana et al. 2021). On the contrary, other studies indicate that the prognosis of elderly-onset epilepsy is generally favorable and DRE is uncommon (Bartolini et al. 2022).

The identification of risk factors and predictors associated with DRE at different ages may contribute to provide appropriate treatment. The main risk factors or predictors for DRE during childhood are early onset of epilepsy (Karaoglu et al. 2021; Kalilani et al. 2018; Xue-Ping et al. 2019; Choi et al. 2020; Kharod et al. 2019; Berg et al. 2012; Wirrell 2013), abnormal neuroimaging or electroencephalography (Berg et al. 2012; Kalilani et al. 2018; Karaoglu et al. 2021; Kharod et al. 2019; Sillanpaa and Schmidt 2009; Sultana et al. 2021; Wirrell 2013; Xue-Ping et al. 2019; Yuan et al. 2018); Seizure type and frequency: focal epilepsy (Berg et al. 2012; Vucetic Tadic et al. 2020), status epilepticus (Karaoglu et al. 2021; Xue-Ping et al. 2019; Yuan et al. 2018), high frequency of seizures (Karaoglu et al.

2021; Sillanpaa and Schmidt 2009), no response to the first ASM (Berg et al. 2012; Mangunatmadja et al. 2021; Mohanraj and Brodie 2013; Sillanpaa and Schmidt 2009), and late remission (Kharod et al. 2019). Other factors include metabolic-genetic disorders (Vucetic Tadic et al. 2020); cognitive deficits or mental disorder (Berg et al. 2012; Karaoglu et al. 2021; Sillanpaa and Schmidt 2009; Sultana et al. 2021). Several studies have shown that there is a higher risk of DRE when seizures occur during the neonatal period compared to the onset of epilepsy later in life (Berg et al. 2012; Choi et al. 2020; Fray et al. 2015; Kalilani et al. 2018; Karaoglu et al. 2021; Kharod et al. 2019; Sillanpaa and Schmidt 2009; Xue-Ping et al. 2019).

Predictive models are helpful tools in the identification of DRE. The presence of two or more predictors increases the probability of DRE. The most common predictors include the age of seizure onset (<12 months), developmental delay at the moment of epilepsy diagnosis, neuroimaging abnormality, and type of epilepsy (Wirrell 2013; Geelhoed et al. 2005). It has been suggested that the seizure type most related to DRE in infants are focal seizures and *status epilepticus* compared to other seizure types (Karaoglu et al. 2021; Vucetic Tadic et al. 2020). In adulthood, it has been established that the most common predictors of DRE include younger age of epilepsy diagnosis, longer evolution of epilepsy, and the presence of more than one seizure per month (Roy et al. 2019). According to the type of seizures, the predictors include complex partial seizures (Roy et al. 2019) and focal epilepsy (Hernandez-Ronquillo et al. 2018; Denton et al. 2021; Gilioli et al. 2012). Other predictors have been proposed, for instance the lack of response to the first ASM (Denton et al. 2021), idiopathic epilepsy (Choi et al. 2020, 2016), neurological comorbidities (Roy et al. 2019; Choi et al. 2020; Hernandez-Ronquillo et al. 2018), intellectual disability (Choi et al. 2016), and EEG abnormalities (Gilioli et al. 2012). In this regard, a prospective study performed in 95 adult patients with DRE showed that the lack of response to the first ASM was the main risk factor of DRE (Denton et al. 2021). Similarly, another study of 1155 adult patients with focal epilepsy showed that 57.8% of them had DRE with similar EEG abnormalities (Gilioli et al. 2012).

18.3 Impact of DRE Throughout Life

The impact of DRE extends far beyond seizures. It promotes the presence of conditions such as intellectual disability (Berg et al. 2012), severe psychosocial consequences (Wirrell 2013), poor quality of life, and sudden unexpected death in epilepsy (SUDEP) (Wirrell 2013; Baranowski 2018). Children with DRE are more likely to suffer developmental disorders. A study involving 198 children (aged <8 years) diagnosed with epilepsy showed that uncontrolled seizures impair the cognitive function (Berg et al. 2012). On the other hand, in adults with DRE, seizure frequency, medical and psychiatric comorbidities, number of SMs, and medication side effects have been correlated with poor quality of life (Baranowski 2018). Szaflarski et al. (2006) studied the impact of the age of seizure onset and duration

of epilepsy on quality of life in patients with DRE. The results showed that age of onset and duration had significant detrimental effects on quality of life. The association of age of onset and duration of DRE with quality of life is explained by mood states and adverse events, which are stronger predictors of quality of life (Szaflarski et al. 2006).

18.4 Age and DRE Treatment

Clinical evidence shows that patients who do not respond to two antiseizure medications (ASMs) have reduced possibilities of controlling their seizures with ASMs (Mohanraj and Brodie 2006; Kwan et al. 2010). On the other hand, aggressive early treatments or appropriate nonpharmacological treatments are recommended for seizure control in infants and young children (Berg et al. 2012; Brodie et al. 2012; Kharod et al. 2019; Mohanraj and Brodie 2013). Timely diagnosis and the correct choice of ASMs play a crucial role in controlling seizures and reducing the generation of other comorbid conditions. A study performed in 1098 patients diagnosed with epilepsy followed their condition up to 26 years. It was found that 49.5% of patients remained seizure-free for at least one year on their first ASM, while 13.3%, 3.7%, 1.0%, and 0.4% of the cohort became seizure-free on the second, third, fourth, and fifth ASMs, respectively. However, since a few patients achieved sustained seizure freedom with the fourth and even the seventh medication regimen, patients who failed with the first three ASD regimens did not become refractory (Brodie et al. 2012). Another study proposed that children with early neuroimaging abnormalities should be seriously considered for surgical evaluation (Wirrell 2013). Similarly, a study reports that 59% of children diagnosed with DRE during the first five years after epilepsy onset remained intractable until epilepsy surgery (Geerts et al. 2012). In particular cases, the DRE treatment includes focal surgical resection and 60–70% the children became seizure-free (Wirrell 2013).

The implementation of ketogenic diet (KD) especially in pediatric patients has been shown to decrease up to 50% the number of seizures (Klepper et al. 2020). Pong et al. (2012) studied 87 patients who met the standard clinical criteria for glucose transporter deficiency syndrome type I (Glut1DS) with a range of age from 3 months to 46 years. Results showed that 90% of patients were confirmed to have epilepsy. The 82% of patients with epilepsy were treated with a KD 1–3 months after diagnosis. From the patients with active seizures at the beginning of the treatment with KD, 83% maintained seizure freedom after stopping preexisting ASMs (Pong et al. 2012). Age also appears to affect treatment outcome, with a higher seizure-freedom rate among older people than younger. Mohanraj and Brodie (2006) conducted a study in 780 teenagers and adults diagnosed with epilepsy to determine the number of ASMs that failed. In the elderly, a remission success rate of 85% was obtained, while in adolescents it was 65% (Mohanraj and Brodie 2006).

18.5 Involvement of Gender and Hormones on DRE

The influence of gender on the predisposition to the development of seizures and epilepsy has been suggested in numerous investigations. A systematic review and meta-analysis performed by Kotsopoulos in 2002 showed a lower incidence of epilepsy in females than males (46.2 versus 50.7/100,000) (Kotsopoulos et al. 2002). The higher incidence in males was attributed to higher exposure to risk factors such as head injury, stroke, and infection of central nervous system (Kotsopoulos et al. 2002). Likewise, the influence of menstrual cycle phases on seizure susceptibility is well known (Bazan et al. 2005). The menstrual cycle also plays a critical role in the success of pharmacological treatment. For example, antiepileptic drugs that exert their actions modulating GABAergic neurotransmission are successful in most male patients and have a poor effect in female patients because their efficacy changes depending of her menstrual cycle (Maguire et al. 2005; Smith et al. 2007).

Even though it seems that females are more resistant to the development of seizures, there are sex differences in terms of the efficacy of pharmacological treatment. Currently, there is no convincing evidence to establish the role of gender in the development of DRE. Kalilani et al. (2018) performed an extensive review to estimate the incidence, prevalence, and risk factors for the development of DRE in male and female patients (Kalilani et al. 2018). The authors concluded that gender is not a risk factor for the development of DRE (Kalilani et al. 2018).

Specific gender-based differences as differential neuronal inhibitory activity has been described (Facciolo et al. 2000; Ravizza et al. 2003). Specifically, females have an increased neuronal GABA activity compared with males. This increase is mediated by an overexpression of the $\alpha 1$ subunit GABA observed in the neurons of the substantia nigra pars reticulata of females (Facciolo et al. 2000; Ravizza et al. 2003). The differences in the inhibitory activity could modify the physiological properties and response to the antiepileptic drugs.

In addition, it has been shown that steroid hormones could modulate the neuronal activity and modify the seizure susceptibility (Reddy 2010). Several reports show that steroids modulate the inhibitory activity mediated by GABA receptors in the brain (Belelli et al. 1989; Bianchi et al. 2002; Reddy and Rogawski 2002). Individually, testosterone has bimodal effects on the seizure genesis (Reddy 2008). The proconvulsive or anticonvulsive effects depending on the animal model and the seizure type (Pesce et al. 2000; Reddy 2004, 2008). Studies in mice demonstrated that the pretreatment with testosterone-derived neurosteroid 3 α -androstenediol, protected against seizures induced by GABA_A receptor antagonist components such as pentylenetetrazol, picrotoxin, and B-carboline ester. However, it has no effect on the seizures induced by Kainic acid, 4-aminopyridine and N-methyl-D-aspartate, which acts as a glutamate receptor agonist (Reddy 2004). On the other hand, studies in humans and animal models have demonstrated that the biphasic effect of testosterone on seizures is mediated by metabolism of estrogens (Herzog et al. 1998; Reddy 2008). Similarly, steroid hormones such as progesterone and deoxycorticosterone

can exert antiseizure actions that are attributed to their conversion to the neurosteroids allopregnanolone (3 α -hydroxy-5 α -pregnane-20-one) and allotetrahydrodeoxycorticosterone (3 α ,21-dihydroxy-5 α -pregnan-20-one; THDOC), respectively (Reddy 2010). The neurosteroids cross the blood–brain barrier and are potent positive allosteric agonists of synaptic and extrasynaptic GABA_A receptors (Chisari et al. 2010; Hosie et al. 2007; Saalman et al. 2007). Also, female rats showed relative resistance to the development of SE induced by pilocarpine during the estrus stage compared to males (Scharfman et al. 2005). The mechanism of resistance to muscarinic cholinergic agonists, such as pilocarpine, in females may be related to the reduction of muscarinic receptors in the hippocampus observed on ovariectomized rats (Cardoso et al. 2010). The treatment with 17 β -estradiol in ovariectomized rats reduces the concentrations of [3H]inositol phosphate, which is increased by muscarinic receptor activation (Pereira et al. 2008). Likewise, muscarinic receptors are lower during the estrus compared with diestrus cycle (van Huizen and Tonnaer 1993). This compilation of evidence suggests that steroid hormones have an effect on neurophysiological activity and seizure susceptibility, and therefore, should play an important role in the gender predisposition to develop DRE.

In addition to hormone-related effects, metabolic differences may play an important role in the gender response to antiepileptic drugs. Ibarra et al. 2013 analyzed the pharmacokinetics sex related differences of the ASM, valproic acid. In this study, females without contraceptive therapy showed a longer lag time, a lower valproic acid hepatic output, and a higher reabsorbed fraction than males. This effect was reverted after a contraceptive therapy (Ibarra et al. 2013). Marino et al. 2012 studied the metabolism of carbamazepine in humans. The authors report a lower absolute clearance and longer half-life compared with females after 100 mg infusion of carbamazepine (Marino et al. 2012). However, it must be considered that these studies were conducted in healthy patients.

18.6 Evidence of the Coexistence of Comorbidities and DRE

The concept of comorbidity has been defined as the occurrence of one or more additional disorders that coexist with a primary condition, in this case, epilepsy (Keezer et al. 2016; Ravizza et al. 2017). It is known that in patients with epilepsy and those with DRE, comorbidities contribute to worsening of the quality of life, increase the severity, and aggravate the prognosis of epilepsy (Kanner et al. 2010; Kanner 2016; Mazarati et al. 2018). The study of this complex relationship is essential; however, it remains barely understood.

Epidemiological studies have shown that the prevalence of psychiatric disorders is higher in people with epilepsy than in the general population (Perini et al. 1996; Gaitatzis et al. 2004c). For instance, a study compared the prevalence or frequency of anxiety and depression in patients with epilepsy (Kwon and Park 2014). The data showed that 9–36% of patients were diagnosed with depression and 11–25% with anxiety. These results indicate that almost one-third of them suffer from depression

and anxiety; nevertheless, these conditions are often underrecognized and undertreated by physicians (Kwon and Park 2014). Correspondingly, other studies showed that mood disorders, followed by anxiety disorders (AD), are the most frequent psychiatric disorders in people with epilepsy (Ettinger et al. 2004; Gaitatzis et al. 2004c,a; Tellez-Zenteno et al. 2007).

The prevalence of epilepsy and its neuropsychiatric comorbidities in older adults is generally higher compared to younger ages (Brodie et al. 2009). A study conducted in 79 subjects, 50 to 67-year-old patients with DRE who underwent epilepsy surgery were followed during 2–16 years. The results showed that 58% of patients were seizure-free, and neuropsychological impairments were identified in 13 patients in the form of depression and loss of concentration (Lang et al. 2018).

Epilepsy may coexist with multiple comorbidities (Medel-Matus et al. 2017). For example, a multiple morbidity was found in a study of 52 patients with DRE who underwent epilepsy surgery. Before and up to 2 years after surgery, all the subjects completed questionnaires in order to evaluate the presence and degree of depression, anxiety, and anger. Eighty-one percent of the patients were seizure free 1 year after surgery. Both anxiety and anger decreased significantly compared to the baseline, while depression showed a slow but nonsignificant reduction. The authors attribute the discrete progression of depression to the fact that many people face difficulties in reorganizing their life even after seizures have disappeared (Meldolesi et al. 2007). Another study evaluated psychopathology in 24 pediatric patients before and 2 years after epilepsy surgery. Psychiatric disorders such as mainly attention deficit and hyperactivity disorder or autism spectrum disorders (ASD) were found in 70.8% of patients before surgery. There were improvements in psychosocial functioning in 66% of the patients 2 years after surgery. None of the seizure-free subjects showed worst psychosocial functioning (Danielsson et al. 2009).

A recent study evaluated the efficacy of the vagus nerve stimulation (VNS) on autistic behaviors in pediatric patients with DRE (Wang et al. 2022). VNS is an extensively used therapy for patients over 4 years old with DRE (Wang et al. 2021b, 2022; Starnes et al. 2019). It has been shown that children candidates for this procedure are at higher risk of behavioral comorbidities, including ASD, compared with children in the general population (Reilly et al. 2014; Sansa et al. 2011). The results showed that the VNS reduced autistic behaviors in the patients, suggesting for the first time the benefits of the VSN treatment for children with DRE and ASD (Wang et al. 2021b). A similar study analyzed the effect of the responsive neurostimulation (RNS) therapy in patients with ASD and DRE. The RNS works by targeting one or two seizure foci in the brain with bursts of stimulation in response to detected abnormal electrographic activity. Sixty-three percent of the patients had a >50% seizure reduction, with 21% of them being classified as super responders (seizure reduction >90%). Improvements in ASD behaviors were reported in 79% of the patients. Based on these results, the RNS system could be a new surgical option for the treatment of people with ASD and DRE (Fields et al. 2022). Additionally, other studies have demonstrated that this therapy also helps in the

rehabilitation of stroke, tinnitus, traumatic brain injury, spinal cord injury, and posttraumatic stress disorder (Hays 2016).

Evidence has shown that, patients with epilepsy have specific features of personality compared to the general population; for example, dysfunctional personality patterns (Novais et al. 2019), a high level of aggression (Shehata and Bateh Ael 2009) and neuroticism (Rivera Bonet et al. 2019). The assessment of personality changes in patients with DRE after successful surgical treatment showed a reduction in the presence of pathological personality traits and neuroticism (Novais et al. 2019; Witt et al. 2008). In harmony with these findings, a recent study evaluated the changes of personality DRE patients who underwent surgical treatment, compared to a control group in one-year follow-up (Iurina et al. 2021). The results revealed that personality traits in patients with DRE changed following surgery. A higher agreeableness was the most relevant difference between the surgical and control group. Furthermore, the surgical group became less neurotic, while the control group increased in the consciousness scale (Iurina et al. 2021).

The association between DRE and its comorbidities has been demonstrated based on the positive effect of the epilepsy surgery reducing comorbid disorders (Lang et al. 2018; Meldolesi et al. 2007; Danielsson et al. 2009). However, some studies expose the effect of the treatment of a comorbidity on DRE, for example, the psychosis therapy in a patient with DRE (De Benedictis et al. 2013). Focal onset epilepsy with impaired awareness seizures accompanied by psychosensorial and psychotic symptoms is a rare condition with no specific treatment (Devinsky 2004; Qin et al. 2005). It was reported the case of a 21-year-old man diagnosed with schizophreniform disorder. The EEG showed an important subcortical epileptic activity with negative response to medication. Symptoms of this type of epilepsy were significantly improved using a psychotherapeutical treatment used for patients with psychotic disorders, known as integrated psychological therapy (De Benedictis et al. 2013). These findings suggest that psychotherapy may be a new treatment modality for patients with this type of DRE (De Benedictis et al. 2013).

It has been shown a higher risk of suicide in patients with DRE (Andrade-Machado et al. 2015; Andrijic et al. 2014). The suicide risk and sleep quality in a cohort of 50 patients diagnosed with DRE were evaluated (Castro et al. 2018). All patients had a neurologic and psychiatric assessment to identify suicide risk, sleep quality, emotional sensitivity, in terms of anger, fear, anxiety, and impulsivity. The results showed that suicidal patients have poor sleep quality compared with nonsuicidal subjects. Similarly, patients with suicide risk showed an increased emotional sensitivity compared to the patients without suicide risk. These findings are important because sleep quality and psychiatric symptoms are rarely evaluated in patients with DRE (Castro et al. 2018). Similarly, sleep disorder has been studied as a comorbidity of DRE in experimental animal models. In particular, *Kcna1* global knockout mice have multiple characteristics associated with DRE (Deodhar et al. 2021). The pharmacological characterization of *Kcna1*-null mice suggests a degree of drug resistance comparable to the human DRE. *Kcna1*-null mice have comorbidities of which DRE is a risk factor, such as cognitive memory deficits (Scantlebury et al. 2017), a sleep disorder that gradually worsens with age (Iyer

et al. 2018), cardiac and respiratory anomalies, and SUDEP (Moore et al. 2014; Iyer et al. 2020, 2018; Simeone et al. 2018; Dhaibar et al. 2019).

18.7 Pathogenic Mechanisms Associated with DRE and Its Comorbidities

The association between DRE and psychiatric disorders could be the result of the existence of shared pathogenic mechanisms (Kanner 2012; Nogueira et al. 2017). Studies in animal models have shown that some pathogenic mechanisms of mood and anxiety disorders facilitate the development of seizures (Kanner 2012; Kumar et al. 2007; Vezzani et al. 1999; Hashimoto et al. 2007; Rajkowska et al. 2007). There is evidence of different types of common mechanisms between psychiatric disorders and epilepsy including endocrine disorders, neuroinflammatory processes, disturbances of neurotransmitters, and mechanisms activated by higher levels of stress (Nogueira et al. 2017). Furthermore, most recent studies propose an important effect of the adenosine deficiency and the localization of the epileptic foci in the coexistence between DRE and its comorbidities.

The endocrine disturbances produced by epilepsy include a high serum concentration of cortisol as a result of a dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis (Kumar et al. 2007). Similarly, depression affects the HPA axis (Mazarati et al. 2009). The effect of the administration of corticosterone in rats has been analyzed as well. The treatment with corticosterone produced a significant reduction in the number of electrical stimulations necessary to reach a fully kindled status compared to the controls (Kumar et al. 2007). Altogether, these findings suggest that the HPA axis is important in both DRE and depression.

The presence of inflammatory cytokines in the CNS during epilepsy also affects the HPA axis and participates in the modification of the expression of neurotransmitters (De Simoni and Imeri 1998) and neuropeptides (Scarborough et al. 1989). Some studies suggest that the inflammatory response from microglia activation by cytokines contribute to the development of psychiatric disorders. For example, it has been shown that the administration of IL-1 β in the hippocampus of rats increases the seizure duration (Vezzani et al. 1999).

It is well known that glutamate is the major excitatory neurotransmitter in the cerebral cortex and plays an important role in mental disorders (Goff and Coyle 2001; Dager et al. 2004; Sanacora et al. 2004; Hashimoto et al. 2007). A study analyzed the level of glutamate in postmortem brains from patients with mental disorders. The authors observed that the levels of glutamate in the brain tissue of major depression and bipolar disorder were significantly higher than control subjects (Hashimoto et al. 2007). Correspondingly, disturbances in the levels of glutamate have been identified in patients with DRE (Sazhina et al. 2020; Wang et al. 2021a).

It has been proven that long-term exposure to stressors affects negatively on mental health (Carroll et al. 2001). The stress response is mediated by the HPA axis

(Herman et al. 2016). Experimental data supports the connection between stress and depression in the epileptic brain. In a rat model of comorbid depression and epilepsy, it has been detected the presence of an overactive HPA axis, with high serum corticosterone levels (Mazarati et al. 2009). Other research showed that the levels of stress, depression, and anxiety were significantly higher in patients with DRE compared to control groups (Moon et al. 2016).

18.8 Localization of Epileptic Foci as a Link Between DRE and Psychiatric Comorbidities

Numerous studies have suggested that the connection between DRE and other comorbid disorders may be due to the localization of the epileptic foci; however, the findings obtained so far are contradictory (Tang et al. 2012; Sperli et al. 2009; Filho et al. 2011). A study performed in 540 adult patients assessed the presence of anxiety and its association with different types of epilepsy. The results showed an independent association of anxiety symptoms with focal epilepsy versus generalized epilepsy (Munger Clary et al. 2018). On the contrary, in another study, 144 patients evaluated for epilepsy surgery received psychiatric examination, and it was found that psychotic syndromes were linked to a history of febrile convulsions and left-sided temporomesial epileptogenic foci (Hellwig et al. 2012). Allebone and collaborators are pioneers in the study of this complex theme. For instance, the existence of a relationship between a dysfunction in the self-identity in patients with chronic focal epilepsy has been shown (Allebone et al. 2015). The authors found a poor self-identity development in patients with seizures arising from a focus in the mesial temporal compared to non-mesial temporal seizure foci (Allebone et al. 2015). Furthermore, the same group found that in patients with epilepsy with mesial temporal foci, objective verbal memory dysfunction, neuroticism, and memory complaints (Rayner et al. 2020). Another study revealed that patients with temporal epilepsy had a higher prevalence of psychiatric comorbidities, predominantly anxiety, than patients with extra-temporal epilepsy (Jansen et al. 2019). Nevertheless, further studies are necessary to identify which disorders of the wide variety of psychiatric comorbidities of DRE can be linked with the localization of the epileptic foci.

18.9 Adenosine Hypothesis of Comorbidities

This hypothesis suggests that adenosine deficiency can be a sufficient cause of an extensive variety of neurological conditions in patients with epilepsy (Boison and Aronica 2015). In the brain, adenosine performs two different roles. As a homeostatic regulator adenosine exerts an inhibitory and neuroprotective effect via activation of

the inhibitory A1 receptors (Meghji and Newby 1990). Conversely, on the synaptic level, adenosine facilitates synaptic function via activation of stimulatory A2A receptors (Cunha 2008).

Adenosine homeostasis in the brain is mainly under the control of metabolic clearance through adenosine kinase (ADK) expression in astrocytes (Boison et al. 2013). Disruptive changes in adenosine homeostasis occur during epilepsy and similarly in other neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Boison and Aronica 2015).

Regarding epilepsy, it has been demonstrated that adenosine is an endogenous antiseizure agent of the brain (Boison 2012; Dragunow 1991). The antiseizure effects of adenosine are mediated mostly via activation of pre- and postsynaptic adenosine A1 receptors (Chen et al. 2013; Boison and Aronica 2015). It has been shown that the increase of adenosine is an important therapeutic approach against epileptic seizures, including those that are refractory to ASMs (Gouder et al. 2003; Guttinger et al. 2005; Huber et al. 2001).

Therapeutic adenosine increase represents a well-validated strategy for the suppression of epileptic seizures in numerous animal models of epilepsy, for instance, focal implant- or cell-based adenosine augmentation prevented seizures in rodents using different models of TLE (Boison et al. 2002; Gouder et al. 2003; Guttinger et al. 2005; Huber et al. 2001; Li et al. 2009; Szybala et al. 2009; Williams-Karnesky et al. 2013). It is important to mention that therapeutic adenosine increment suppressed carbamazepine-resistant seizures in the intrahippocampal kainic acid model of TLE (Gouder et al. 2003). Alternatively, dietary intervention such as therapy with a high-fat low-carbohydrate "ketogenic diet" (KD) is known to increase adenosine in the brain (Masino et al. 2011). KD therapy is effective in preventing seizures in epilepsy and promotes cognitive function improvement (Freeman 2009; Lutas and Yellen 2013).

With regard to DRE, a study focused on children with pharmacoresistant epilepsy, analyzed the effect of a modified Atkins diet, which is a less restrictive variation of the KD, the results showed that 30% of children experienced 90% seizure reduction and 52% of children experienced 50% seizure reduction after 3 months (Chen et al. 2019; Sharma and Jain 2014). On the other hand, the using the KD in adults, 32% of the patients with DRE showed >50% seizure reduction (Caraballo et al. 2017; Chen et al. 2019).

Furthermore, KD therapy is considered for the treatment of other neurologic disorders associated to DRE such as Alzheimer's disease (Aso et al. 2013; Van der Auwera et al. 2005), Parkinson's disease (Cheng et al. 2009), and amyotrophic lateral sclerosis (Zhao et al. 2006).

Pharmacological adenosine kinase (ADK) inhibition is another approach for adenosine augmentation (Tescarollo et al. 2020). The ADK is the major metabolizing enzyme of adenosine (Boison 2013). In the adult brain, ADK is predominantly expressed in astrocytes (Studer et al. 2006). There is a pathological upregulation of ADK expression in the epileptic brain of animal models and humans (Aronica et al. 2011). It has been confirmed that pharmacological inhibition of ADK with

5-iodotubercidin suppresses seizures in the KA model of DRE in mice (Gouder et al. 2004). Even though the vastly efficient effect on seizure reduction, including those resistant to common ASDs (Gouder et al. 2003). However, although they have shown high efficacy in suppressing seizures, ADK inhibitors are currently in very limited clinical use due to cardiovascular side effects of systemic adenosine elevation (Boison 2013; McGaraughty et al. 2005).

18.10 Perspectives and Opportunities

DRE represents a challenge due to the complexity and diversity of the mechanisms associated. While in the preceding years notable advances in the neuroscience field have occurred, such as improvements in diagnostic techniques and more pharmacological therapies, the epidemiological investigations indicate that these improvements are not reflected in broad benefits for patients with DRE (Tellez-Zenteno et al. 2014). Patients with DRE have to deal with multiple difficulties on their search for a solution for their devastating condition. For example, approximately 5% of patients with DRE enter in a seizure remission period that can last for years, which provides these patients with a false hope (Callaghan et al. 2011).

For years, different hypotheses have been proposed attempting to explain the possible causes of DRE in order to develop more effective therapeutic options for patients (Fig. 18.1). The main hypotheses include those mechanisms generated by overexpression of transporters in peripheral organs such as the gut, liver, and kidney (Lazarowski et al. 2007); seizure-induced neuronal network degeneration that inhibits access of ASMs to neuronal targets (Fang et al. 2011; Rogawski 2013); variations in genes associated with pharmacokinetics and pharmacodynamics of SADs producing inherent drug resistance such as changes in voltage-gated ion channels and neurotransmitter receptors (Tang et al. 2017); and the relationship between epilepsy severity and pharmacoresistance (Rogawski 2013).

According to the different hypotheses triggering DRE, and considering clinical and experimental evidence, it is plausible the idea that the relationship of DRE with age, gender, and comorbidities is a multifactorial. Therefore, none of the hypotheses proposed so far can separately explain the DRE. Consequently, it is essential to find a relationship between diverse hypotheses and how they complement each other to understand all the variables that affect to people with DRE. In this context, it is essential to establish a global hypothesis that integrates all the phenomena associated with the drug resistance in epilepsy (Pérez-Pérez et al. 2021).

Even though, at this time there is no solid evidence regarding to establish the role of gender in the development of DRE. Some investigations have indicated that gender is not a risk factor for the development of DRE (Kalilani et al. 2018). However, there are inconsistencies in terms of definitions and classifications of DRE, for which these results should be interpreted carefully and according to particular considerations.

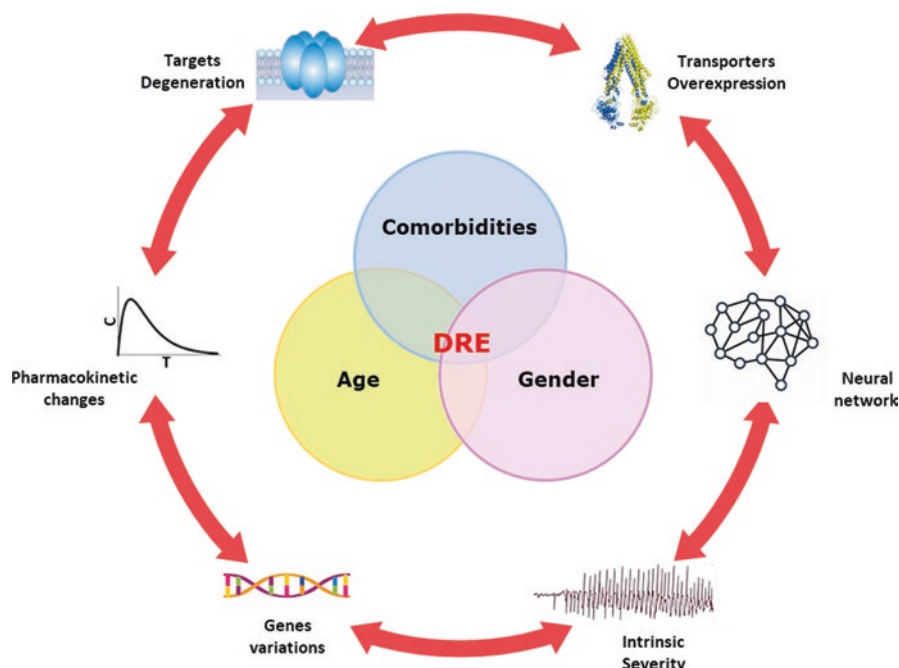


Fig. 18.1 Overview of proposed hypotheses for possible underlying mechanism(s) of antiseizure drug (ASD) resistance. (1) The *Pharmacokinetic Hypothesis* proposes that overexpression of drug efflux transporters in peripheral organs decreases ASD plasma levels, thereby reducing the amount of ASD available to enter the brain and reach the epileptic focus. (2) The *Neuronal Network Hypothesis* states that seizure-induced degeneration and remodeling of the neural network suppresses the brain's seizure control system and restricts ASDs from accessing neuronal targets. (3) The *Intrinsic Severity Hypothesis* proposes that common neurobiological factors contribute to both epilepsy severity and pharmacoresistance (30). (4) The *Gene Variant Hypothesis* states that variations in genes associated with ASD pharmacokinetics and pharmacodynamics cause inherent pharmacoresistance. These genes include metabolic enzymes, ion channels, and certain neurotransmitter receptors that are targets for ASDs. (5) The *Target Hypothesis* postulates that alterations in the properties of ASD targets, such as changes in voltage-gated ion channels and neurotransmitter receptors (e.g., GABAA receptor), result in decreased drug sensitivity and thus lead to refractoriness. (6) The *Transporter Hypothesis* states that overexpression of ASD efflux transporters at the blood–brain barrier in epilepsy leads to decreased ASD brain uptake and thus ASD resistance

Psychiatric comorbidities have been associated with reduced quality of life, increased disability, increased medication, and medical costs in persons with DRE. Furthermore, it has been revealed that the age of patients is also an important factor that determines the type of comorbidity related to epilepsy. In children with epilepsy, the most prevalent comorbidities include attention-deficit hyperactivity disorder, mood and anxiety disorders, and autism spectrum disorder (Hamiwka et al. 2011). On the other hand, disorders such as depression and anxiety disorder are frequently observed in adults (de Boer et al. 2008). These neuropsychiatric

comorbidities in patients with DRE are yet highly underdiagnosed and undertreated. The correct identification and treatment of comorbidities in DRE are critical because approximately two-thirds of premature deaths in patients with epilepsy are attributed to comorbid diseases (Gaitatzis and Sander 2004).

A deeper examination of each patient with DRE must be performed by physicians and neurologists in order to identify particular factors that may be related with either the joint development of neuropsychiatric comorbidities or the ineffective response to medication. There is clear evidence that shows that patients with a family psychiatric history are more likely to develop pathologic reactions to stressors (Kanner 2014). Particularly, family psychiatric history plays an essential role in drug resistance (Kanner 2014; Mula et al. 2003), unfortunately it is frequently under evaluated. In this context, a retrospective study of individuals with DRE explored the presence of mental and physical diseases one-year prior to the diagnosis of epilepsy (Teneralli et al. 2021). The authors found that 45% of the patients with DRE were diagnosed with a general mood disorder, followed by depression (41%), anxiety (38%), and drug dependence (38%). All of the most common psychiatric diagnoses were higher among the patients with DRE compared with the patients without DRE. These findings emphasize the importance of an early and efficient identification of neurological disorders (Teneralli et al. 2021).

Regarding the relevance of age on epilepsy, it has been revealed that both epilepsy and its neuropsychiatric comorbidities are most frequent in elderly people (Lang et al. 2018). A limited number of studies have compared characteristics of epilepsy in older adults versus younger individuals (Grivas et al. 2006), and even less is known about this matter in DRE. Certainly, the aged population is rapidly growing mainly in developed countries, therefore more studies focused on the mechanistic processes of DRE, alone or in combination with other neurological disorders, on each stage of life are needed with the purpose of generating specific treatments and or preventive actions.

18.11 Conclusions

It is essential a better understanding of DRE from a comprehensive approach, as a dynamic relationship between the different mechanisms influenced by numerous inherent variables of a living organism such as age, gender, and comorbid disorders (Fig. 18.1).

The diagnosis and management of the psychiatric comorbidities in patients with DRE represent a critical factor in order to reduce their impact on the development of epilepsy and improve their response to the treatment. Even though some mechanisms connecting DRE and comorbidities have been proposed, additional investigation is yet needed in order to clarify this relationship.

Finally, a deeper understanding of the molecular and neural network basis of age and sex differences in seizure and drug response is essential to design new effective sex- and age-specific therapies for DRE.

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Chapter 19

Indications for Intracerebral Recording in Candidates for Epilepsy Surgery



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Abstract Epilepsy surgery depends on accurate preoperative localization of the epileptogenic zone (EZ). Then, presurgical evaluation is necessary to obtain the most accurate information from clinical, anatomic, and neurophysiologic aspects, with the final goal of performing a personalized surgical treatment. The noninvasive methods of seizure localization and their results must be interpreted in conjunction, to achieve establish localization hypotheses of the anatomic location of the EZ. This chapter presents evidence supporting that Stereoelectroencephalography is an extraoperative invasive method useful to anatomically define the EZ and the related functional cortical areas of patients with medically refractory focal epilepsy.

Keywords Stereoelectroencephalography · Epilepsy surgery · Refractory focal epilepsy

19.1 Introduction

Epidemiological studies from developing countries suggest a prevalence of drug-resistant epilepsy comparable to that in developed countries (Palmini 2000; Singh and Sander 2020). Epilepsy surgery is an important treatment option for people with drug-resistant epilepsy (Wiebe et al. 2001) and that alleviates the extremely difficult situation of the patients and their families. A recent review has demonstrated that epilepsy surgery is available in few low- and middle-income countries and is even

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less common when intracerebral monitoring is needed for definition of the epileptogenic zone (EZ) (Singh and Sander 2020).

The noninvasive methods of seizure localization, including videoelectroencephalogram (video-EEG) and imaging, must be interpreted in conjunction, to establish the anatomical localization of the EZ, according to the temporal and spatial organizational pattern of the epileptic activity. When the noninvasive data are insufficient to define the EZ, extraoperative invasive monitoring could be indicated. Stereoelectroencephalography (SEEG) is an invasive method of exploration for drug-resistant focal epilepsies, contributing to a three-dimensional and temporally precise study of the epileptic discharge. SEEG allows to define the EZ and the related functional cortical areas, the anatomo-electrical correlations, and tailored surgeries (Talairach et al. 1974; Trébuchon and Chauvel 2016; Munari et al. 1994; Bancaud et al. 1970; Talairach and Bancaud 1973).

This method was designed and developed in the 1960s in Paris, France, by Talairach and Bancaud (Isnard et al. 2018). The concept of EZ was created by the pioneers of this method in order to identify the region where the seizure originates and also distinguish the anatomical distribution of the interictal activity from the lesion. Identification of the EZ using SEEG has gained acceptance over the last decade in contrast to electrocorticography (McGonigal et al. 2019).

The history of modern medicine shows that technological advances are initially expensive and therefore limited to countries with high income levels. As their use expands, the costs of new technologies decrease, making them more accessible. As with other technologies, the use of SEEG has begun to be used in developing countries. In our experience, the creation of local specialized epilepsy centers capable of performing presurgical evaluation and epilepsy surgery faces several challenges, most importantly the need to support well-trained people who can evaluate and operate patients, including awareness and critical analysis of technological advances; with relatively limited resources.

We present our experience in Argentina since 2014. Our Surgical Epilepsy Center was active in a public hospital from 1984 to 2014. In 2014, the professional team of this center moved to another public hospital, continuing its research and educational activity. Some members of the Surgical Epilepsy Center began to perform acute intraoperative SEEG in 1984, with its inclusion as chronic extraoperative recording procedure in 1994.

According to our experience, we demonstrated that surgical treatment associated with SEEG of patients with drug-resistant epilepsy can be performed in our country, with similar outcomes as in developed countries. The procedure includes different phases that are described below.

19.2 Noninvasive Phase

In this phase, the challenging goal is the localization of the EZ using noninvasive techniques. The procedures include video-EEG recording, which associated with the clinical findings, allow to identify the cortical structure of the hypothetical networks that may be involved in seizure organization (Giagante et al. 2003). Additional validation of the anatomical hypothesis is accomplished through structural imaging, the identification of lesion using 3-Tesla magnetic resonance imaging (MRI) (Princich et al. 2013; Blenkmann Phillips et al. 2017), and positron emission tomography (PET) (not always available in our Center). In addition, this phase includes neuropsychologic and mental health evaluation.

The noninvasive studies facilitate the localization of the EZ in most of patients undergoing presurgical evaluation. However, in some patients, a clear and unique hypothesis is not possible. In such cases, the noninvasive phase does not allow the epileptologists to decide between different hypotheses. When the noninvasive data are insufficient to define the EZ, invasive monitoring may be indicated.

19.3 Invasive Phase, SEEG

19.3.1 *Criteria for Indication of SEEG*

The invasive studies such as SEEG are indicated due to failure to localize the EZ with noninvasive methods because either (1) the EZ is suspected to involve extratemporal areas, (2) MRI is negative, (3) bilateral onset is suspected, (4) bilateral lesion, i.e., hippocampal sclerosis, is observed, or (5) MRI findings do not correlate with scalp Video-EEG monitoring.

The epileptogenic network is not limited to the dimension of the lesion or by its clinical expression. Even if the discovery of a lesion statistically comes with a higher probability of cure, the SEEG is based on the concept of epileptogenic network and must follow the rule of independence among lesional, interictal, and ictal information.

19.3.2 *Methodology: Acquisition, Recording, and Analysis*

SEEG methodology follows the criterion originally described by Bancaud et al. (1970), which is based on anatomo-electro-clinical correlations (AECs). The implantation strategy is personalized, with electrode placement based on preimplantation hypotheses take into account each seizure, electroclinical correlations, and if there is relation with a suspected lesion.

SEEG is carried out during long-term monitoring (7 to 12 days/24 h). Indeed, Video-SEEG recording can be carried out as long as necessary in order to record several of the patient's seizures.

The protocol during the ictal and postictal periods includes systematic patient assessment performed by qualified technical staff. During the recording process, patients are instructed to promptly advise the staff whenever they experienced their first symptom associated with the seizure activity.

Changes in video-SEEG recordings can be correlated with symptom and its precise time of onset and associated ictal activity.

Six to twelve multilead electrodes (Ad Tech) are implanted per patient, in temporal and extra temporal areas, depending on the suspected origin and region of early spreading of seizures. Electrodes are implanted perpendicular to the midline vertical plane with the patient's head fixed in the Talairach stereotactic frame. For mesial temporal lobe structures, depth electrodes are inserted through 2.5 mm diameter drill holes, using orthogonal orientation, usually targeting the amygdala, and anterior and mid/posterior hippocampus locations. These electrodes are implanted through the middle or inferior temporal gyrus and provide recordings from gyral and adjacent sulcal cortices (from superficial contacts) and mesial structures (deepest contacts).

When the hypothesis of EZ involves extratemporal areas, other electrodes are implanted to localize the EZ, its extension to these regions, or to study the seizure propagation. The extratemporal areas include parietal perisylvian regions, the orbitofrontal cortex, the operculo-insular region through the frontal operculum, the temporal operculum, anterior or posterior cingulate, and Heschl gyrus. Indeed, oblique trajectories are used to reach the postero-medial orbitofrontal cortex.

The characteristics of each electrode are as follows: (a) 9 or 10 platinum contacts with intercontact distance of 2.6 (Spencer) or 4.43 (Micro-Macro) mm, contact length of 2.41 mm and 1.1 mm diameter (Spencer), or (b) 9 platinum contacts, 1.43 mm distance between the first and the second contact, 4.43 mm interelectrode distance from the second to the last, contact length of 1.57 mm, and 1.28 mm diameter.

19.4 Experience of Argentine Epilepsy Surgery Program and SEEG

In the program of epilepsy surgery from Argentina (Oddo et al. [2021](#)), the signals are recorded using a 128-channel Micromed, Brain Quick SD LTM Model 64 express, filtered between 0.3 and 7500 Hz, and sampled at 30,000 Hz. The SEEG signal is low-pass filtered at 1000 Hz, sampled at 2000 Hz, and recorded using the a 128-channel EEG.

The exact position of each electrode is verified by immediately post implantation computed tomography images (CT) fused with a previous MRI (Princich et al.

2013). The electrodes are left implanted up between 7 and 12 days until sufficient information is obtained, including the recording of typical seizures.

EEG activity is displayed using bipolar recordings between contiguous contacts and/or referential montages, in a global montage and in more selected channels, grouping adjacent and tightly connected regions and displaying them along rostrocaudal and/or dorsoventral axes. In addition, the electrocardiogram is systematically monitored.

All SEEG recordings are reviewed by 3 qualified neurologists experienced in video-EEG interpretation. Consensus about EEG changes is achieved by the 3 experts after their discussion.

Recordings are reviewed daily to identify background and paroxysmal activity. The SEEG analysis includes the identification of the ictal onset pattern as well as interictal activity. For interictal analysis, periods of 2 h of EEG recording are evaluated during day, night, and sleeping time. The presence of slow EEG activity, spikes, spike and wave, and fast discharges is investigated. Evaluation of ictal activity includes the identification of seizure onset indicated by changes from the background leading to a clear seizure discharge without returning to the basal activity. The seizure onset zone (SOZ) was identified on SEEG recordings as the region where the following activity occurred at the very onset of the clinical seizure or before the earliest ictal manifestation: 1) low voltage fast activity; 2) a high-amplitude slow synchronizing wave with fast activity superimposed on the ascending or descending slope of the slow wave; and 3) repetitive spikes or sharp waves in the preictal phase, followed by fast activity (Figs. 19.1, 19.2 and 19.3).

19.4.1 Electrical Stimulation During SEEG

Seizures triggered by electrical stimulation (ES) have important diagnostic value when they reproduce the usual clinical pattern of the patient's seizures. Bipolar ES is carried out to map the eloquent areas and to evaluate the epileptic threshold in the epileptogenic cortex. If necessary, antiseizure medication is progressively tapered during SEEG monitoring (Trébuchon and Chauvel 2016; Chauvel et al. 2019; Collavini et al. 2021).

ES is usually performed after the recording of spontaneous seizures, around day 4–5 of the SEEG. It is carried out using two contiguous contacts of each electrode, applying bipolar and biphasic current stimulation. Single bipolar pulse or train of stimuli are used to induce ES. The presence of after-discharges evoked by ES helped to differentiate epileptogenic from nonepileptogenic areas.

Two different protocols can be used to induce ES:

- Low-frequency ES (shock stimulation): pulses of 0.3 to 3 ms of duration at 0.5 to 5 mA, applied at 1 Hz during 20 to 60 s.
- High-frequency ES (train stimulation): pulses of 0.1 to 1 ms of duration at 0.5 to 5 mA, applied at 50 Hz during 5 s.

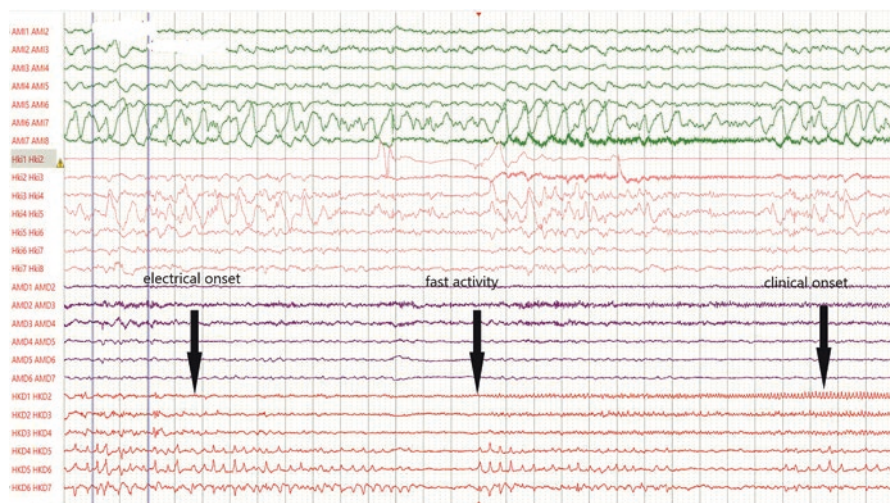


Fig. 19.1 First and second arrows indicate a sustained low-voltage fast activity pattern. Third arrow indicates the onset of the clinical seizure manifestation associated with cognitive changes

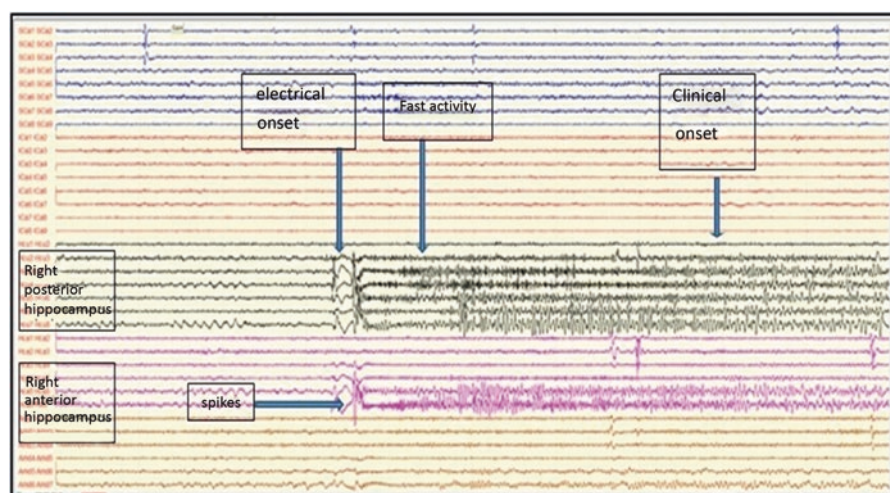


Fig. 19.2 The first two arrows indicate a high-amplitude slow synchronizing wave with fast activity superimposed, followed by a fast activity (second arrow). Third arrow indicates the onset of clinical seizure manifestations associated with epigastric sensation

Changes in brain function can be evaluated after ES is applied, evaluating different tasks such as naming, automatic speech (counting), repetition, reading aloud, and fluency.

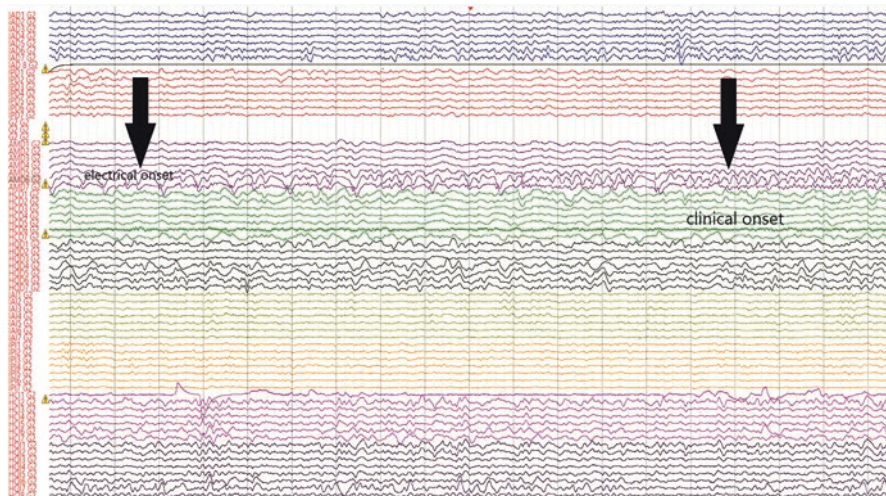


Fig. 19.3 The two arrows indicate repetitive spikes and sharp waves, progressing to a high amplitude fast discharge. These discharges correlate with the clinical onset of the seizure associated with humming sensation and auditory illusions

19.4.2 MRI CT Acquisition and PET

Our program involves the imaging evaluation of all patients. It includes MRI, in a Philips Achieva 3T Magnet Unit, with final in-plane isotropic resolution of 1 mm (TR/TE/TI = 9.2/4.2/450 ms, matrix 256 Å~ 256, bandwidth 31.2 kHz, FOV 256 mm Å~ 256 mm, and 180 slices). We perform subject-specific anatomical segmentation, skull stripping, and extraction of 3D pial surfaces for further evaluations using the freesurfer image analysis (<http://surfer.nmr.mgh.harvard.edu/>). Noncontrast CT (LSVCT GE, 64 detectors) scans are performed for each patient immediately after implantation of electrodes in order to visualize IEs contacts and as part of the clinical evaluation.

For each patient, we obtain the high-resolution preoperative T1 MR and post-implanted CT, using 3D slicer open source medical image analysis platform (<http://www.slicer.org>). This procedure assured that the CT images and IEs are aligned with preimplant MRI and all of the cerebral parcellations and 3D brain reconstructions provided by freesurfer as described in details by Princich et al. 2013 (<https://www.frontiersin.org/articles/10.3389/fnins.2013.00260/full>).

Our hospital does not have PET equipment. However, in some patients, we are able to perform interictal F-fluorodeoxyglucose (FDG) PET.

19.4.3 Neuropsychological Evaluation

The neuropsychological protocol used in our epilepsy surgery program is the same that previously published by our group and proved to be useful to reinforce the hypothesis of EZ (Oddo et al. 2003; Lomlondjian et al. 2011; Allegri et al. 1997; Munera et al. 2014; 2015).

Neuropsychological evaluation is performed in three different stages: Before surgery, 6 months after resection and a year afterwards.

The protocol contains tasks to evaluate the following functions: (i) Forward and Backward Digit Span, WAIS, and Trail Making Test Part A to evaluate attention; (ii) Rey Auditory Verbal Learning Test (RAVLT) and List Learning Test to evaluate verbal memory; (iii) Rey-Osterrieth Complex Figure Test (RCFT) to analyze visual memory; (iv) Wisconsin Card Sorting Test (WCST), Trail Making Test Part B and Verbal Fluency (FAS) to evaluate executive function; (v) Boston Naming Test (BNT), and Token Test (TT) to evaluate language; (vi) Intelligence quotient (IQ); (vii) Edinburgh Questionnaire (EHQ) to evaluate handedness; and (viii) Hooper tests to estimate judgment.

19.4.4 Psychiatric Assessment

The psychiatric assessment is performed by trained psychiatrists, during video-EEG scalp monitoring (which usually lasts five days) according to a standardized protocol, with two main objectives: diagnosis of comorbidities and psycho-education for surgical treatment or invasive procedures (D'Alessio et al. 2014; Scévola et al. 2017; Lombardi et al. 2021). If the patient has a seizure, the interview is interrupted until the postictal period ended. Psychiatric semiology is supplemented with structured interviews for present and past history of psychiatric disorders and personality disorders using standardized psychiatric assessments-the Structured Clinical Interview for Axis I diagnoses of DSM-IV (Structured Clinical Interview for DSM Disorders [SCID]-I and -II, respectively) (First et al. 1999). In addition to these interviews, specific scales are used: Global Assessment of Functionality (GAF) (American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders 2000), Beck Depression Inventory (BDI II) (Beck et al. 1996), and Barratt Impulsiveness Scale Factor structure of the Barratt impulsiveness scale (Patton et al. 1995). Also, the psychological evaluation includes McGill Illness Narrative Interview Schedule (MINI) (Groleau et al. 2006), Brief Illness Perception Questionnaire (Broadbent et al. 2006), Quality of life in epilepsy-31 P inventory (Cramer et al. 1999).

19.4.5 Surgery and Follow-up Evaluation

The surgical plan is finalized principally based on the results of the ictal SEEG and supported by results from other noninvasive evaluations, which helped to identify the EZ.

Then, the patients are submitted to the epilepsy surgery, and electrode removal is carried out in the operating room.

All treatment recommendations are made according to the discussion carried out by the multidisciplinary epilepsy group. Surgery is performed in the same institution by the same surgeons (P.S and E.S.). A histopathological examination of the resected brain tissue is performed for all operated patients. Postoperatively, patients are followed up by the surgeon (P.S.), as well as by epileptologists, neuropsychologists, psychiatrists, and psychologists (Oddo et al. [2021](#)). Epilepsy surgery outcome is estimated according to the ILAE classification system. Good and poor outcomes are defined as ILAE classes 1 to 3 and 4 to 6, respectively (Wieser et al. [2001](#)).

19.5 Basic Anatomic Functional Organization EZ Hypothesis Located in Patients with Temporal Lobe Epilepsy

Basic knowledge about anatomic circuitry is frequently considered in the SEEG planning. In patients with hypothetical EZ in mesial temporal lobe structures, depth electrodes are implanted in orthogonal fashion in adjacent amygdaloid complex and hippocampus (head, body and tail) in order to better define their involvement (or not). The implantation plan depends primarily on patient semiology and secondarily on the results of MRI. In addition, electrodes are implanted in the perirhinal and entorhinal cortex. When the EZ hypothesis exceeds the mesial temporal lobe and considers that the amygdaloid complex has important connectivity with other brain areas, SEEG includes the anterior perisylvian areas, the rostral portions of the superior temporal gyrus (planum polare), the orbital frontal cortex. Orthogonal lateral electrodes are implanted when it was necessary to evaluate the anterior insular, limen insulae, subcallosal cingulate areas, and frontal opercular areas. Thus, even if the phase no invasive hypotheses are pointing to the frontal parietal or occipital lobe, with presence of a lesion or not, our group preferred to investigate the associated limbic cortices.

19.6 Results of Our Experience

In our experience (Oddo et al. [2021](#)), between 2014 and 2021, we performed 273 standard anterior temporal lobectomies plus amygdalohippocampectomy (SATL+AH) and a total of 50 SEEGs. We selected 21 patients who underwent

SEEG, with a main EZ hypothesis located in the temporal lobe. Fourteen patients (14/21, 66.6%) were found eligible for surgery. Eight patients underwent a standard anterior temporal lobectomy plus amygdalo-hippocampectomy (SATL+AH), in the dominant hemisphere that included 3–3.5 cm of resection from the temporal pole, whereas in nondominant cases, the resection was 4 cm from the temporal pole (left $N = 3$, right $n = 4$). In addition, one patient underwent a right temporal lobectomy extended to Heschl gyrus. One patient underwent a frontotemporal corticectomy, one patient had temporal resection with anterior insulectomy, and one patient had a temporal resection and orbito frontal corticectomy.

Because of the pandemic, three patients remain on a list for surgery. Seven patients were formally excluded from surgery because of the bilateral (seizures originating independently or concomitantly in both temporal lobes) or multifocal origin of their seizures.

None of the patients presented post-surgical complications.

19.7 Conclusion

The use of SEEG monitoring in patients with temporal lobe epilepsy has specific indications, following the French guidelines on SEEG and TLE (Isnard et al. 2018). With mesial symptomatology or lesion, SEEG is indicated when the extralimbic or extratemporal cortex, or the contralateral temporal lobe seem to show early involvement, according to the non-invasive data. SEEG is also indicated in the absence of radiological features of hippocampal sclerosis, or when noninvasive explorations suggest extratemporal origin or extension, or a bilateral epileptogenic zone.

The central step in SEEG is to define a strategy for electrode placement. The integration of presurgical video-EEG, especially ictal clinical semiology, neuroimaging, and neuropsychological data, allows us to define the EZ hypothesis and plan the SEEG scheme.

For this instance, a well-trained multidisciplinary team that includes staff epilepsy neurologists, psychiatrists, neuropsychologists, psychologists, neuroradiologists, and neurosurgeons constitutes the fundamental pillar.

We considered essential work with a very strict protocol, evaluating only patients who clearly are candidates of receiving surgical treatment and underwent SEEG. A recent paper from an epilepsy center in the United Kingdom (Singh and Sander 2020) reports that only half of the adult patients with drug-resistant focal epilepsy who undergo presurgical evaluation, proceed with surgery. Our SEEG electroclinical findings are similar to those described by other authors (Chauvel et al. 2019; Di Vito et al. 2016; Feng et al. 2020; Ferrari-Marinho et al. 2016). Clinical semiology plays a fundamental role for the implantation scheme, and electrical activity demonstrates the neural correlates during the evolution of the seizure. The stereotyped electroclinical patterns contribute to the definition of the EZ and to knowledge about temporal lobe networks and their anatomical correlates. Identification of

certain semiology sequences indicates their anatomical correlations and the probable ictal trajectories in the brain in temporal and extra temporal seizures (Chauvel et al. 2019). In our experience, surgery was indicated in 14 (66.6%) patients, which is similar to the proportion described in other epilepsy centers around the world (Palmini 2000; Wiebe et al. 2001; Chauvel et al. 2019).

Despite the development of strong preimplantation hypotheses, some epilepsy localizations may be associated with bilateral EZs. There is controversy about surgical treatment in patients with bilateral temporal lobe epilepsy. Didato et al. (2015) concluded “that surgical treatment should be discarded in patients with bilateral temporal lobe epilepsy (BTLE), as identified on ictal scalp during video-EEG.” In a meta-analysis of published data on surgical outcomes in 1403 patients with presumed BLTLE, Aghakhani et al. (2014) reported, “when SEEG confirmed the bilaterality of seizures (“true BTLE”), by showing independent right and left seizures, only 45% of patients had a good outcome after unilateral temporal lobectomy.” In our study, patients with bilateral independent seizure onset identified by SEEG were not considered for surgical treatment, considering the poor outcome reported in those reports. Two other patients were excluded for surgery due to the multifocal origin of their seizures.

An important aspect to consider in countries with limited economical resources like ours is the high direct cost of surgery, especially when it SEEG is necessary. However, to date, there is no other therapeutic alternatives for this group of patients, so we believe that SEEG should be included in the diagnosis protocols of all the epilepsy centers of the world. In our center, we conducted a study of direct costs of hospitalization in the video-EEG Unit, conventional surgery, and SEEG, resulting in an approximate 4500 US dollars per patient (Pereira de Silva et al. 2016). Recent studies in developed countries demonstrated that surgical intervention is compared with nonintervention and the cost-effectiveness of SEEG (Garcia-Lorenzo et al. 2019; Kovács et al. 2021). Other authors have evaluated the direct costs of different SEEG techniques and carried out a cost-effectiveness analysis, estimating a total cost of 8000 Euros per patient. It is important to consider that the high costs of these procedures and treatments are significantly offset by the improvement in patient outcomes.

Based on the results presented in this chapter, we hope to encourage the expansion of epilepsy surgery centers in the region, define the technical requirements, and, despite the high costs of the electrodes and technical equipment, raise the possibility of their development with the same quality as that carried out in developed countries (Palmini 2000). We expect that, as with other technological resources, the increased use of own resources will encourage their development, with the same level and technical security as in developed countries, but with a significant reduction in costs.

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Chapter 20

On the Development of New Drugs for the Treatment of Drug-Resistant Epilepsy: An Update on Different Approaches to Different Hypotheses



Alan Talevi

Abstract Despite the continuous expansion of the available pharmacological options for the treatment of epilepsies and remarkable advances in understanding their pathophysiology, the proportion of refractory patients has remained roughly unchanged over the past 100 years.

In the last decade, hypotheses that try to explain the drug-resistant phenotype have increased in number and their scope has been more precisely specified, and some major advances related to some of these hypotheses have been realized, both at the preclinical and clinical levels. These include the use of gene therapies to revert the pharmacoresistant phenotype in animal models of epilepsy, advance into clinical trials and approval of tailored multitarget therapeutics (e.g., padsevonil and cenobamate) exhibiting encouraging results on refractory patients, approval of new drugs with new (and sometimes complex) mechanisms to address particularly severe and difficult-to-treat epileptic syndromes, and the first reports of applications of network analysis to rationally select combinations of antiseizure medications. The introduction of the *Epilepsy Therapy Screening Program* also constitutes a significant milestone that will possibly have a major impact on the development of new, more efficacious therapeutic options against epilepsy, as the focus of the international guidelines to screen for novel medications against epilepsy is now on refractory epilepsy and disease-modifying interventions.

This chapter, which intends to be a critical update of the one published back in the first edition of this volume, overviews the current hypotheses that intend to explain refractory epilepsy as well as plausible therapeutic strategies to address some of them.

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Keywords Refractory epilepsy · Drug-resistant epilepsy · Pharmacoresistant epilepsy · Intractable epilepsy · Antiseizure medications · Multitarget drugs · Transporter hypothesis · ABC transporters · Target hypothesis · Drug discovery · Drug design · Network pharmacology · Nanocarriers · Epilepsy

20.1 Drug-Resistant Epilepsy: Possible Explanations

According to the current definition of the International League Against Epilepsy (ILAE), the term *drug-resistant epilepsy* (often used interchangeably with intractable, pharmacoresistant, or refractory epilepsy) refers to “the failure of adequate trials of two tolerated and appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom” (Kwan et al. 2010). Several dimensions must be examined when considering this definition. First, “adequate trials” implies that the therapeutic intervention has been applied at adequate strengths for a sufficient length of time. “Appropriately chosen” denotes that the chosen intervention has previously been demonstrated to be effective, preferably through randomized controlled trials, for the patient’s epilepsy and seizure type. These aspects of the definition are not trivial at all. For instance, a pharmacological intervention that has been inappropriately selected according to the type of epilepsy will not be counted as one of the two (appropriate) interventions required by the definition before concluding that the patient is, in fact, drug resistant. Similarly, when a drug is withdrawn because of an adverse event before it has been titrated to its clinically effective dose range, thus not constituting an “adequate trial,” it will not be counted as one of the specified interventions. These considerations are not only of utmost importance when deciding how a patient’s disorder will be managed but also from a drug discovery perspective: some therapeutic interventions that could possibly achieve the target seizure-free status might be disregarded due to poor tolerability. Therefore, although the focus of this chapter is on therapeutic interventions addressing the underlying cause of pharmacoresistance, the development of new drugs for drug-resistant epilepsy should not exclude the need for safer, better-tolerated medications. It should also be noted that, at present, the terms antiseizure medications (ASMs) or antiseizure drugs (ASDs) are preferred over antiepileptic drugs to describe those pharmacological interventions that, in essence, are intended for symptomatic control (which does not exclude the possibility of beneficial effects on the course of the disease and comorbidities that result from downstream effects of seizures) but that have not demonstrated direct favorable actions on the underlying disease or its progression (Perucca et al. draft).

An increasing number of hypotheses have been raised to explain the origin of drug-resistant epilepsy (Tang et al. 2017; Bazhanova et al. 2021): the highly inter-related *transporter* and *pharmacokinetic hypotheses* (Löscher and Potschka 2005; Tang et al. 2017); the *target hypothesis* (Löscher and Potschka 2005; Schmidt and

Löscher 2005; Kwan and Brodie 2005; Remy and Beck 2006); the *gene variant hypothesis* (which may converge with the transporter, pharmacokinetic, and target hypotheses, as discussed later) (Cárdenas-Rodríguez et al. 2020); the *epigenetic hypothesis* (Kobow et al. 2013); the *intrinsic severity hypothesis* (Rogawski and Johnson 2008); the *neural network hypothesis* (Fang et al. 2011); and the *neuroinflammation hypothesis* (Löscher and Friedman 2020; Campos-Bedolla et al. 2022).

The *transporter hypothesis* suggests that drug resistance may arise from acquired activation or overexpression of efflux drug transporters that restrict drug distribution to the brain and/or parenchyma cells; such over-expression could occur at any of the cells of the neurovascular unit. The *pharmacokinetic hypothesis*, in essence complementary to the previous one, considers the role of efflux transporters outside the brain, and also the possible contribution of other drug clearance mechanisms, that is, biotransformation enzymes, to the insufficient bioavailability of ASMs. Research supporting the transporter hypothesis has focused on efflux transporters from the ATP-binding cassette (ABC) superfamily. Cumulative studies have revealed high expression levels of members of this superfamily, such as P-glycoprotein (Pgp), the breast cancer resistance protein (BCRP), and multidrug resistance protein (MRP), at the neurovascular unit of nonresponsive patients with epilepsy, either at the blood–brain barrier (BBB), glial cells and/or neurons (see, for instance, Tishler et al. 1995; Dombrowski et al. 2001; Sisodiya et al. 2002, 2006; Aronica et al. 2003, 2005; Lazarowski et al. 2004; Calatozzollo et al. 2005; Kubota et al. 2006; Ak et al. 2007). The lack of efficacy of those ASDs which are substrates of any of the upregulated efflux transporters could therefore be a consequence of the limited brain bioavailability of ASD (Marchi et al. 2005). However, in other cases, refractoriness has not been related to subtherapeutic concentrations specifically at the site of action, but to persistently low plasma levels of the drug due to enhanced plasma clearance, despite the administration of standard doses of ASMs (Lazarowski et al. 2004, 2007; Iwamoto et al. 2006; Czornyj et al. 2018). This could be related to the high expression levels of efflux transporters in other organs (e.g., intestines, liver, and kidney), which would restrict absorption and facilitate elimination (Tang et al. 2017). A valid question would be whether a therapeutic intervention can be regarded as an “adequate trial,” thus contributing to the diagnosis of intractable epilepsy, if the chosen dose could not achieve therapeutic plasma concentrations. As exposed by Tang and collaborators (op. cit.), while it could be argued that abnormalities in ASD plasma concentrations would be readily captured by therapeutic drug monitoring, reference therapeutic plasma concentrations are not expected to be universally applied, and from a precision medicine perspective, it would be possibly better to define the target plasma concentration and to adjust ASD dosages accordingly on an individual basis. Interestingly, a series of recent articles by Lazarowski et al. have provided grounds for the suggestive theory that overexpression of Pgp outside the brain may be causally related to heart failure and sudden unexpected death in epilepsy (Auzmendi et al. 2018, 2021; Akyuz et al. 2021; Czornyj et al. 2022), which, if confirmed, would add a new size to the pharmacokinetic hypothesis.

A general pharmacokinetic mechanism underlying drug-resistant epilepsy is consistent with the fact that the available ASMs act through a wide range of pharmacological targets. The transporter hypothesis has been fully validated in preclinical models of epilepsy. High levels of Pgp, associated with low brain bioavailability of its substrates, have been observed in animals with drug-resistant epilepsy, and the resistant phenotype has been reversed by co-administration of Pgp inhibitors (van Vliet et al. 2006; Brandt et al. 2006; Zhang et al. 2012). Conclusive evidence of the validity of the transporter hypothesis in humans, however, remains elusive, and the author's perspective is that interest in this hypothesis has diminished to some extent in recent years (possibly following disappointing clinical trials with second- and third-generation Pgp inhibitors in the field of oncology (Chung et al. 2016)), although some of the more recently proposed hypotheses provide mechanistic insight on how the increased expression of drug transporters is induced and regulated.

There are anecdotal cases (Summers et al. 2004; Ianetti et al. 2005; Schmitt et al. 2010; Pirker and Baumgartner 2011) and small-scale open-label studies (Asadi-Pooya et al. 2013; Narayanan et al. 2016) that showed improvement in patients with drug-resistant epilepsy when ASMs were co-administered with verapamil, a known Pgp inhibitor, but it is unclear whether the observed results are due to the intrinsic antiseizure activity of verapamil, Pgp inhibition, other effects on the drug pharmacokinetics, or more than one of these reasons. Using positron emission tomography (PET) and the PET ligand and Pgp-substrate (R)-[11C] verapamil with and without tariquidar (a selective Pgp inhibitor) in pharmacoresistant patients, Feldmann et al. (2013) corroborated the association between regionally localized Pgp overactivity and drug resistance patients with temporal lobe epilepsy. However, a small-scale randomized controlled trial showed no statistically significant decrease in seizure frequency in the pharmacoresistant patients receiving verapamil as adjuvant therapy; only 12 of the recruited patients completed the study (Borlot et al. 2014). Randomized controlled trials with selective inhibitors are needed to obtain definitive proof of the therapeutic potential of this theory.

The main argument against the transporter hypothesis is that, while several ASMs are proven substrates for ABC transporters, others are not (Zhang et al. 2012; Leandro et al. 2019); in fact, the evidence shows that the standard broad-spectrum ASD, valproic acid, is not transported by ABC carriers (Baltes et al. 2007; Leandro et al. 2019). As the transporter hypothesis has not been convincingly validated in clinical trials, current guidelines for the management of epilepsy do not consider the interaction with ABC transporters as a criterion for medication choice (Kanner et al. 2018; Park et al. 2019; Guery and Rheims 2021).

The *target hypothesis* proposes that compositional/structural (transcriptional or posttranscriptional) acquired alterations in the pharmacological targets of ASDs might explain the drug-resistant phenotype (Fattorusso et al. 2021; Fonseca-Barriendos et al. 2022). This hypothesis is based on reported loss of sensitivity to voltage-gated sodium channel blockers such as carbamazepine and phenytoin in patients and animal models of epilepsy (Schmidt and Löscher 2009). It has been observed that the inactivation effect of phenytoin on sodium channels is transiently

reduced in kindling models (Vreugdenhil and Wadman 1999), whereas the use-dependent effects of carbamazepine and phenytoin are permanently lost or reduced in the pilocarpine model of epilepsy and in some patients with temporal lobe epilepsy (Remy et al. 2003a, b; Jandová et al. 2006). Numerous changes in the expression of sodium channels subunits have been described in animal models of seizure and epilepsy and in patients with epilepsy (Bartolomei et al. 1997; Gastaldi et al. 1998; Aronica et al. 2001; Whitaker et al. 2001; Ellerkmann et al. 2003), suggesting that epileptogenesis and/or seizures may alter the ASDs targets. Mutations in the accessory subunit $\beta 1$ have been linked to a dramatic loss in the use-dependent effect of phenytoin (Lucas et al. 2005). Furthermore, associations have been reported between alterations in GABAA receptor subunits and resistance to phenobarbital in animal models of temporal lobe epilepsy (Volk et al. 2006; Bethmann et al. 2008). The Achilles heel of the target hypothesis is that clinical ASMs associated with different modes of action exist, and even those ASDs that share a common mechanism (e.g., GABAA receptor allosteric modulators) sometimes bind to different binding sites of the same pharmacological target. Thus, the target hypothesis by itself would only satisfactorily explain the phenomenon of multidrug resistance involving drugs that share their mechanism and would be even less valid to explain resistance to drug combinations. However, as discussed in other sections of this chapter, some novel ASMs based on a multitarget strategy have shown encouraging results in drug-resistant patients. The outcome could be explained through the target hypothesis, but other possible explanations could be offered, as discussed in another chapter.

The *gene variant hypothesis* states that variants of genes involved in the pharmacodynamics and pharmacokinetics of ASMs or associated with the epileptic phenotype could be the source of drug resistance. It is clearly related to the transporter, pharmacokinetics, and target hypotheses, only that it specifies an intrinsic origin of the resistant phenotype rather than an acquired source of variability due to the course of the disorder and/or treatment. For instance, recent studies, including meta-analyses, have suggested an association between polymorphic variants of alpha and beta subunits of voltage-operated sodium channels and differences in their responsiveness to ASMs (e.g., Nazish et al. 2018; Bao et al. 2018; Zhang et al. 2021a; Li et al. 2021). In contrast, the *epigenetic hypothesis* argues that seizures may mediate epigenetic modifications resulting in persistent genomic methylation, histone density, posttranslational modifications, and noncoding RNA-based changes (Kobow et al. 2013). Liu et al. (2016) analyzed DNA methylation across the entire genome in brain tissue from ten drug-resistant patients and demonstrated the presence of several differentially methylated genes on the X chromosome and a significantly smaller number on the Y chromosome. Lv et al. (2019) investigated 75 Chinese patients (25 with CBZ-resistant epilepsy, 25 with CBZ-responsive epilepsy, and 25 controls) and found an association between methylation levels in the EPHX1 promoter and the CBZ-resistant phenotype.

The *intrinsic severity hypothesis* suggests that the inherent severity of the disorder is a key determinant of treatment outcomes (Rogawski and Johnson 2008). Epidemiological studies indicate that the single most important predictor of the

response to pharmacological interventions in epilepsy is the number of episodes at the initial stage of the disorder (MacDonald et al. 2000; Williamson et al. 2006; Sillampää and Schmidt 2006, 2009; Mohanraj and Brodie 2006; Kim et al. 2006; Hitiris et al. 2007). Recently, it has been suggested that the intrinsic severity hypothesis should be expanded to consider not only seizure frequency but also pathological high-frequency oscillations as an indicator of severity (Santana-Gomez et al. 2022). Some shortcomings of the intrinsic severity hypothesis have been underlined in the past (Schmidt and Löscher 2009): the lack of studies on the biological basis of disease severity; the lack of genetic studies comparing patients with low seizure frequency versus patients with high seizure frequency at the onset of the disorder; and the fact that there are reports of nonresponsive patients with low frequency of episodes in the early phase of epilepsy (Spooner et al. 2006). Some of these limitations are now being actively remedied through current sequencing technologies: sequencing-based studies on patients with nonlesional epilepsies have recently identified novel risk genes associated with severe epilepsies and revealed an excess of rare deleterious variation in less severe forms of epilepsy (Epi25 Collaborative 2019, Calhoun and Carvill 2020).

The *neural network hypothesis* states that adaptive remodeling of neural circuits induced by seizures may contribute to the development of drug-resistant epilepsy (Fang et al. 2011). Bearing in mind that remodeling of neural circuits also occurs in responsive patients, differences between the degree of neural reorganization in responsive and nonresponsive patients should be studied to support this latest explanation of drug resistance. In a recent perspective article discussing the need for a complex systems approximation to achieve a better understanding of drug resistance in epilepsy, Servilha-Menezes and Garcia-Cairasco (2022) underlined the fact that the occurrence of comorbid disorders in patients with epilepsy is associated with a negative prognosis regarding the chances of achieving and sustaining a seizure-free status. Interestingly, some common comorbid disorders with epilepsy, such as depression and anxiety, are also associated with abnormal neural networks/circuits (Duval et al. 2015; Oberlin et al. 2022) and, as importantly, with poor response to pharmacotherapy (Oberlin et al. 2022).

Finally, the *neuroinflammation hypothesis* suggests that inflammatory factors released during seizures can induce blood–brain barrier dysfunction (leaky vessels) and compensatory overexpression of efflux transporters, resulting in a loss of response to ASMs. Importantly, changes in microvascular permeability following seizures seemingly result in the increased transport of high-molecular-weight proteins (e.g., albumin), but not necessarily the free exchange of small ions or molecules (Kang et al. 2013). Consequently, unbound, pharmacologically relevant concentrations of ASDs in the brain may diminish (Marchi et al. 2009; Potschka et al. 2011). In other words, sub-efficacious unbound drug levels could arise from both reduced free drug levels due to complexation with albumin and increased expression of efflux pumps.

It is clear from the short precedent overview that refractory epilepsy is a complex, multifactorial phenomenon and that different hypotheses may explain the drug resistance phenomenon in different subgroups of patients (e.g., the gene variant

hypothesis would only apply to patients expressing the gene variants linked to drug resistance), whereas in some patients more than one hypothesis might be integrated to explain the resistant phenotype, or might exhibit some degree of overlap and convergence, as previously discussed by other authors (Schmidt and Löscher 2009; Servilha-Menezes and Garcia-Cairasco 2022). For instance, the transporter and pharmacokinetic hypotheses speak of a seizure- and/or treatment-induced activation of similar clearance mechanisms at the neurovascular unit or in organs outside the brain (e.g., liver and kidneys), whereas the gene variant hypothesis relates the activation of efflux and enzymatic biotransformation systems to genetic polymorphism (e.g., at regulatory regions of a gene). The neuroinflammation hypothesis provides a mechanistic explanation for the acquired overexpression of transporters at the blood–brain barrier and/or epileptic foci, as well as a complementary mechanism to explain reduced, subtherapeutic free drug levels in the brain parenchyma (extravasation of plasma proteins and sequestration of unbound drug).

In the following sections, we discuss some potential or current therapeutic approximations to address some of the previously overviewed hypotheses.

20.2 Possible Therapeutic Answers to the Transporter and Pharmacokinetic Hypothesis

The traditional hypothetical answer to overcome efflux transporter-mediated drug resistance was to develop therapeutic systems capable of evading or ameliorating the active efflux, either by inhibiting or downregulating ABC transporters, by hiding the ASDs from these systems (in a “Trojan horse” manner), or by designing novel ASDs without any affinity for ABC transporters. Potschka (2012) provided an excellent review on this matter.

The general strategies can then be synthesized as follows: (a) modulation of ABC transporters (i.e., inhibition and/or downregulation of transporters), (b) design of novel drugs which are not efflux transporter substrates, and (c) bypassing drug transport (or the Trojan horse strategy).

Most research on these strategies has focused on Pgp, the best-known representative of the ABC superfamily. However, several proteomic studies have shown that, in humans, the levels of BCRP at the neurovascular unit are comparable (if not higher) to those of Pgp (see Table 20.1) (Uchida et al. 2011; Shawahna et al. 2011; Al-Majdoub et al. 2019). Differences between cortical and subcortical tissues have also been observed (Huttunen et al. 2022). Moreover, numerous reports agree that the expression levels of different ABC transporters are interrelated, with direct and inverse co-expression patterns, depending on the case (Bordow et al. 1994; Choi et al. 1999; Cisternino et al. 2004; Bark et al. 2008; Miller et al. 2008). Since there is some degree of overlap across the substrates of different transporters, the possibility of upregulation of a given transporter to compensate for the disturbance of

Table 20.1 Expression levels of different ABC transporters found in brain microvessels across different quantitative proteomic studies (healthy brain tissue)

Transporter	Absolute protein expression levels (pmol/mg total protein)		
	Uchida et al. (2011)	Shawahna et al. (2011)	Al-Majdoub et al. (2019)
Pgp-ABCB1	2.85 (0.58)	3.98 (0.88)	2.58 (0.93)
MRP1-ABCC1	<0.21	<0.21	<0.05
MRP5-ABCC5	<0.50	<0.50	<0.01
MRP6-ABCC6	<0.17	<0.17	0.48 (0.06)
BCRP-ABCG2	6.06 (1.69)	6.15 (1.41)	2.22 (0.61)

another should be considered, especially when pursuing long-term therapeutic interventions, as in the case of epilepsy.

Initially, the inhibition of ABC transporters was intended with adjuvant administration of small-molecule inhibitors, as originally conceived in the field of oncology to deal with chemoresistance. Although nonclinical and initial clinical studies in the field of cancer treatment were promising at first, trials of first-, second-, and even third-generation agents have been terminated mostly due to serious safety issues (Deeken and Löscher 2007; Fox and Bates 2007; Lhommé et al. 2008; Tiwari et al. 2011). At this point, it is important to emphasize that ABC transporters comprise a concerted, complex efflux system with a prominent role in the disposal of waste products and toxins, and they also participate in the traffic of physiological compounds. Thus, permanent impairment or disruption is likely to result in severe side effects (again, one should bear in mind the chronic nature of epilepsy, which requires long-term treatment).

Recent research has focused on elucidating intracellular signaling pathways that control ABC transporters (their expression, intracellular trafficking, activation, and inactivation), such as those dependent on inflammatory stress and the activation of nuclear receptors. It has been proposed that identifying the molecular switches of these transporters will allow selective and transient modulation of transporter activity and/or expression for therapeutic purposes in different clinical scenarios (Hartz and Bauer 2010; Miller 2015), which includes turning the efflux mechanisms off for short, controlled periods. For instance, subchronic treatment with the cyclooxygenase-2 inhibitor SC-58236 blocked the status epilepticus-associated increase in Pgp expression in the lithium-pilocarpine status epilepticus model and enhanced the brain penetration of phenytoin (van Vliet et al. 2010). More recently, using siRNA, Yu et al. blocked inhibitory κ B kinase subunit β (IKK β) gene transcription, which functions as an upstream regulator of inflammation and nuclear factor-kappa B activation (Yu et al. 2014). siRNA targeting IKK β was delivered to rats before seizure induction by kainic acid, abolishing Pgp overexpression and decreasing seizure susceptibility in epileptic rats. Enrique et al. reported a mouse model of drug-resistant seizures based on the subchronic administration of proconvulsant doses of 3-mercaptopropionic acid (Enrique et al. 2017). Reduced sensitivity to known Pgp substrate ASDs (phenytoin and phenobarbital) was observed; such a loss of response was not extended to non-substrates of Pgp, such as carbamazepine, diazepam, or

levetiracetam. Loss of sensitivity was reversed by co-administration of the Pgp inhibitor nimodipine, and Pgp overexpression was observed in the cerebral cortex, hippocampus, and striatum of the animals. This model was later used for screening new drugs capable of reversing the drug-resistant phenotype (Enrique et al. 2021). A virtual screening campaign was implemented with a focus on compounds that could simultaneously elicit anticonvulsant and anti-inflammatory effects. The underlying rationale was that treatment with such multitarget compounds would block Pgp upregulation induced by glutamate and pro-inflammatory signals. Subchronic administration of one of the *in silico* hits, sebacic acid, during the seizure-induction period was able to revert the overexpression of Pgp similarly to celecoxib. Although the anti-inflammatory effects of the virtual screening hits were not validated, this study seems to be conceptually in line with the transporter hypothesis as well as the neuroinflammation hypothesis. A similar study was conducted by Liu et al. (2022) who found that antioxidant preventive treatment with N-acetylcysteine also prevented the development of resistance.

An alternative strategy that could provide delivery of a drug to the brain without the toxic issues associated with the impairment of efflux mechanisms involves the identification of novel ASDs that are not recognized by ABC transporters (Demel et al. 2008, 2009). Such an approximation implies the use of ABC transporters as antitargets. Review articles on the use of structure- and ligand-based approaches to detect substrates for Pgp and other ABC pumps have been published in the past (Klepsch et al. 2014; Montanari and Ecker 2015); more recent studies on the subject have relied on modern machine learning approximations such as adaptive learning (Cerruela García and García-Pedrajas 2018), ensemble learning (Hou et al. 2020), and deep learning (Zhang et al. 2021b), among others. Couyoupetrou et al. (2017) described the implementation of a virtual screening campaign to identify anticonvulsant drugs with no substrate liability for Pgp. Four of the chosen hits were tested in a bidirectional transport assay using an MDCK II- MDR I cell monolayer. The efflux ratios obtained in the presence and absence of amiodarone (a Pgp inhibitor) showed no significant differences, confirming the lack of significant Pgp-mediated efflux at the assayed concentration. Similarly, Gantner et al. (2017) proposed BCRP as an antitarget in a virtual screen exercise and identified four anticonvulsant agents with no affinity for such transporter.

The last strategy oriented to bypassing the biochemical barrier posed by efflux transporters involves the use of a carrier system (e.g., a nanocarrier or a prodrug) to “hide” the drug from the efflux system. Additionally, it should be emphasized that the targeting of nanoparticulated systems might be favored in leaky vessels; accordingly, drug delivery to the brain through pharmaceutical nanocarriers could also be linked to the neuroinflammation hypothesis. Moreover, drug administration via routes or delivery methods that avoid or minimize the first-pass effect or that protect the drug from elimination mechanisms could also be used in relation to the pharmacokinetic and gene variant hypothesis.

A wide variety of nanosystems have been studied to enhance permeability to the brain, especially in the field of oncology, whereas the degree of advancement for other neurological disorders seems to lag slightly (Sim et al. 2020; Hersh et al.

2022). An exhaustive overview of these studies lies outside the scope of this chapter. Regarding the specific application of this strategy to encapsulate ASDs, different nanosystems have been studied for the delivery of clonazepam, diazepam, phenytoin, ethosuximide, 5–5-diphenyl hydantoin, carbamazepine, valproic, oxcarbazepine, phenobarbital, and NMDA receptor antagonists, among others (Fresta et al. 1996; Kim et al. 1997; Jeong et al. 1998; Nah et al. 1998; Darius et al. 2000; Friese et al. 2000; Thakur and Gupta 2006; Abdelbary and Fahmy 2009; Varshosaz et al. 2010; Eskandari et al. 2011; Scioli Montoto et al. 2018, 2021, 2022). A central question would be whether these pharmaceutical technology artifices are capable of improving the bioavailability of drugs in the central nervous system and, if so, the molecular basis of such improvement. Unfortunately, most of these reports limit their scope to the physical characterization and in vitro behavior of the reported systems. Nevertheless, some of them have explored the in vivo behavior with variable results. Darius et al. (2000) found that brain tissue levels of valproic acid were not significantly modified by administration inside nanoparticles, although the nanosystem was found to reduce drug metabolism via mitochondrial beta-oxidation. Friese et al. (2000) reported that poly(butyl cyanoacrylate) nanoparticles coated with polysorbate 80 extended the duration of the anticonvulsive activity of the NMDA receptor antagonist MRZ 2/576, presumably by preventing active transport processes. Eskandari et al. (2011) observed an enhanced protective effect of valproic acid in the maximal electroshock seizure (MES) test when the drug was administered within nanostructured lipid carriers. Intranasal administration of 4 mg/kg of the encapsulated drug led to almost three-fold higher brain concentrations than an intranasally administered solution of 30 mg/kg of valproic acid, and the brain–plasma ratio was also increased through the nanocarrier. Scioli Montoto et al. (2018) reported that protection from seizures by carbamazepine incorporated into a nanostructured lipid carrier remained for at least 2 h after intraperitoneal administration, but there was no difference from the free drug group.

Prodrugs are another option to circumvent the blood–brain barrier, sometimes making use of uptake transporters from the solute carrier (SLC) superfamily (e.g., dopamine is administered as its precursor l-dopa, which is transported into the brain by the l-type amino acid transporter and metabolized to release dopamine in situ) (Mandaya et al. 2010). Numerous prodrugs of different anticonvulsant agents such as phenytoin, gabapentin, valproic acid, and eslicarbazepine have been developed with the goal of improving bioavailability by modifying drug absorption, distribution, and/or elimination (Bennewitz and Saltzman 2009; Trojnar et al. 2004; Bialer and Soares-da-Silva 2012). For example, DP-VPA (Fig. 20.1) was conceived to be specifically activated at the epileptic foci. In it, a molecule of valproic acid is linked to lecithin, leading to a 50-fold increase in efficacy in the pentylenetetrazol-induced seizures test (Trojnar et al. 2004).

Noteworthy, in the last decades, it has been proven that many pharmaceutical excipients which are usually incorporated into pharmaceutical delivery systems can inhibit or modulate ABC transporters' function through different mechanisms (Bansal et al. 2009; Nguyen et al. 2021). For example, it has been proposed that PEG and surfactants, such as sorbitans and polysorbates, can disrupt the lipid

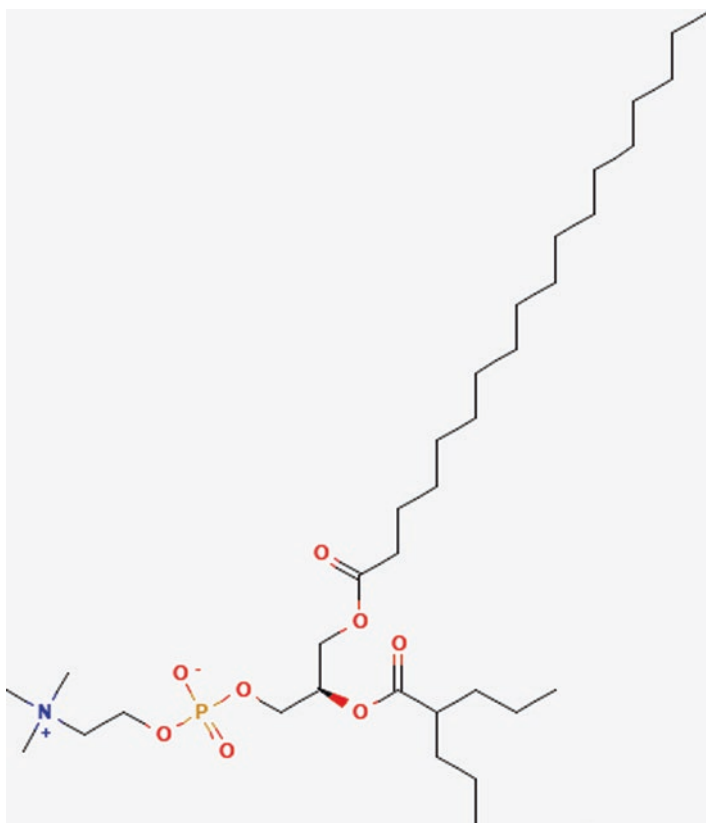


Fig. 20.1 DP-VPA, a prodrug of valproic acid, has been investigated as a potential treatment for severe forms of epilepsy, including status epilepticus, acute repetitive seizures in children, bipolar disorder, and migraine prophylaxis

arrangement of the cellular membrane and that these perturbations have been shown to modulate Pgp activity (Lo 2003). This kind of modulation is interesting because it may increase drug bioavailability in a transient manner, without the undesired effects of direct inhibition. Besides their possible role in modulating transporters, cumulative evidence indicates that nanoparticles' coating leads to the adsorption of elements from the blood, such as apolipoproteins, which in turn would allow distribution to the brain by receptor-mediated transcytosis (Wohlfart et al. 2012 and references therein).

20.3 Possible Therapeutic Answers to the Target Hypothesis

Several (if not most) central nervous system disorders present a complex etiology that includes a combination of polygenic, environmental, and neurodevelopmental factors. Empirical evidence with treatments for mood disorders from the

phenotypic-screening era (e.g., antidepressants) shows that searching for polyspecific, selective nonselective drugs (multitarget-directed ligands, multitarget drugs, polyvalent drugs, hybrid drugs, or “magic shotguns”) may prove a safer and more efficacious way to address such complexity than the development of highly selective, single-target drugs (Roth et al. 2004; Margineanu 2016). There are abundant examples of recent developments in the field of central nervous system therapeutics based on this relatively new paradigm, including drugs in development for Alzheimer’s and Parkinson’s diseases (Cavalli et al. 2008; Youdim and Buccadfasco 2005), depression, schizophrenia, and others (Decker and Lehmann 2007; Wong et al. 2010).

There are many reasons why multitarget therapies are also of most interest within the field of epilepsy. Empiric evidence has suggested that—if total drug load is carefully monitored—some refractory patients may achieve seizure remission on polypharmacy, especially if the pharmacologic properties of the specific ASDs being combined are considered (Canevini et al. 2010; Kwan and Brodie 2006). Second, the recent introduction of ASDs with novel (fenfluramine) or complex (cannabidiol) modes of action has proven successful in particularly resistant, severe, and catastrophic epileptic syndromes, such as Dravet and Lennox-Gastaut (Devinsky et al. 2018; Balagura et al. 2020; Scheffer et al. 2021). Third, many currently used ASDs are unintended multitarget agents selected through phenotypic models of seizures (Bianchi et al. 2009). Fourth, the design of tailored multitarget ASDs sounds like a natural answer to the target hypothesis of drug resistance, considering that it is unlikely that two distinct drug targets will lose sensitivity to drugs simultaneously. The benefits of targeting more than one rationally selected target can also be achieved by drug combinations chosen from a network pharmacology perspective. Combination therapies for epilepsy are covered in a separate chapter of this volume, which deals with epilepsy and complexity.

Two of the most recently developed drugs for refractory epilepsy Refractory epilepsy (RE) are, in fact, tailored multitarget agents. Cenobamate (Fig. 20.2) is a dual agent that acts on voltage-operated sodium channels and as an allosteric positive modulator of the GABA_A receptor. A post hoc analysis of a subset of patients from a long-term multicenter phase 3 open-label study showed high rates of sustained 100% and ≥90% seizure reduction. Almost half of the patients who decided to continue with cenobamate after the study was finalized achieved seizure freedom for at least 12 months (Fig. 20.3) (Sperling et al. 2021). Noteworthy, the patients enrolled in the study had been diagnosed with focal epilepsy and had previously failed to achieve seizure freedom despite being treated with stable doses of 1–3 ASMs.

Encouraging results were also obtained in a phase 2a, randomized, placebo-controlled, double-blind (3 weeks) plus open-label (8 weeks) multicenter study of padsevonil (Fig. 20.4) as an add-on therapy (padsevonil being another dual-acting agent which acts through SV2s and as a partial, low-affinity allosteric modulator of GABAA receptor) (Muglia et al. 2020). The study enrolled refractory patients with focal epilepsy who had failed to control seizures with four or more ASDs regimens of adequate dose and duration. During the blind period, patients in the treatment

Fig. 20.2 Cenobamate: a recently approved dual-acting ASM with encouraging results as add-on therapy in patients with drug-resistant epilepsy

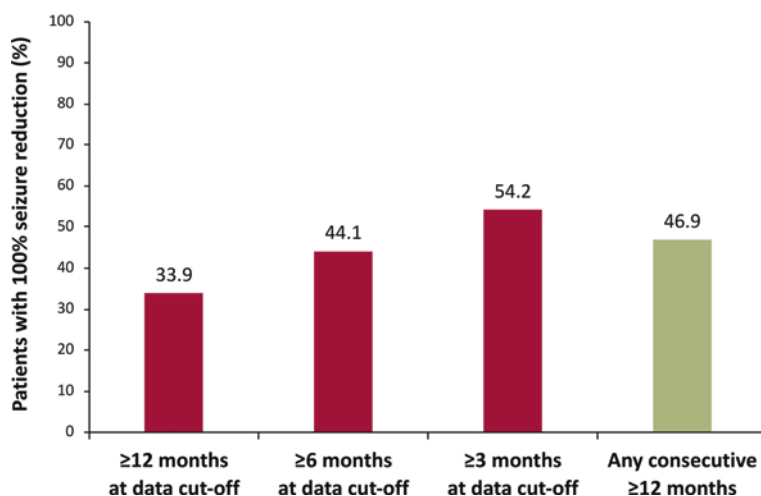
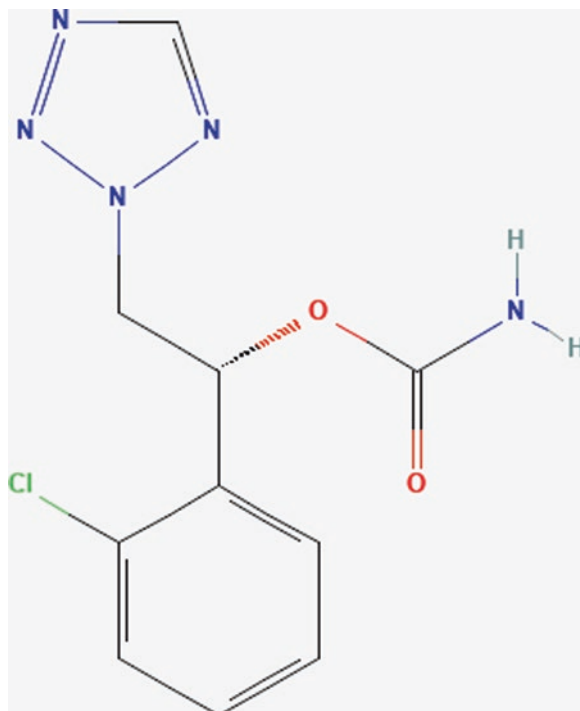


Fig. 20.3 Among the patients that remained on cenobamate as add-on therapy after a phase 3 large-scale open-label study, 46.9% achieved seizure freedom for at least 12 months. (Reproduced from Sperling et al. (2021) under a Creative Commons license)

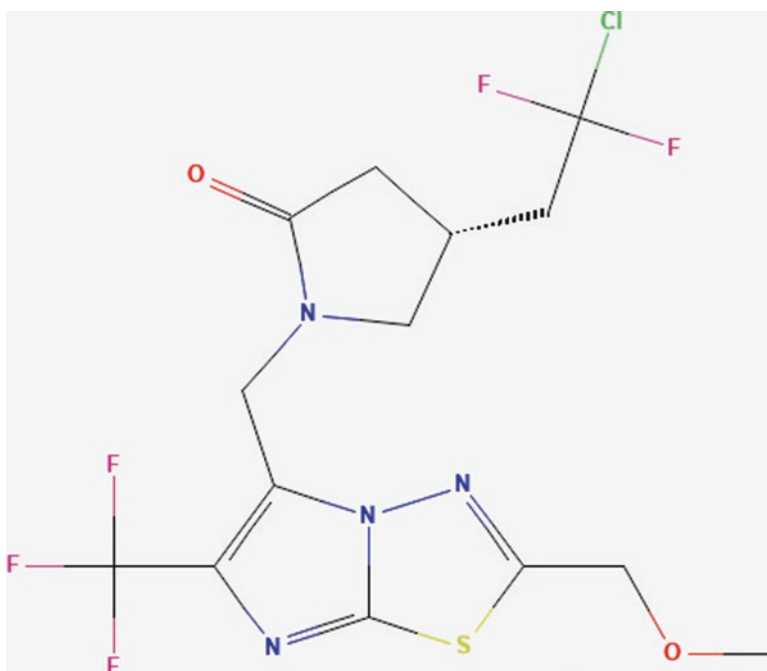


Fig. 20.4 Padsevonil is a dual-acting ASD in development, which has shown promising results in drug-resistant epileptic patients participating in a phase 2a study

group rapidly achieved seizure reduction of approximately 50%, whereas no clear benefit was observed in the placebo group. When switched to treatment, seizure reduction was also observed in the placebo group. Remarkably, 76% of the patients chose to remain on padsevonil treatment after the study ended, reflecting the positive perception on the benefits of the intervention. Later, however, a phase 2b study failed to demonstrate the superiority of padsevonil (Contreras-García et al. 2022).

20.4 Conclusions

In recent years, the number of hypotheses that aim to explain the drug-resistant phenomenon in epilepsy has expanded, and new ideas have expanded the horizon of the classical tentative explanations to the resistant phenotype. It is possible that no single hypothesis may explain all cases of refractory epilepsy, and the available explanations partially overlap and/or converge in many cases.

Among the strategies proposed to cope with drug-resistant epilepsies associated with genetic or acquired upregulation of brain and/or peripheral transporters, drug design of new ASDs with no substrate liability for ABC transporters appears as a reasonably safe option. Circumventing transport by either prodrug design or

encapsulation or conjugation of ASDs with nanodelivery systems seem also as a good alternative. Noteworthy, the neuroinflammation hypothesis of drug-resistant epilepsy suggests that the delivery of pharmaceutical nanocarriers to the brain could be enhanced by passive targeting of the seizure-induced leaky vessels. Considering the physiologic (and critical) role of efflux transporters, downregulating their activity to basal levels should be preferred, due to safety reasons, to fully abolish their function. Interestingly, seizure models that achieve overexpression of efflux transporters at the brain capillaries have been reported and might be of help to screen for novel therapeutics that can prevent or reverse the resistant phenotype.

On the other hand, innovative ASDs with complex pharmacology, in line with a systems biology perspective, have been successfully introduced to the market in the last few years or are under investigation for the treatment, as add-on therapies, of drug-resistant epilepsies, with encouraging results at clinical trials.

The increasing knowledge of how oxidative stress and inflammation contribute to a negative circle (where seizures induce changes that contribute to the occurrence of new seizures) opens new paths to the development of new treatments that might be of special value when facing epilepsies characterized with severe and frequent seizures.

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Chapter 21

Physical Exercise as a Strategy to Reduce Seizure Susceptibility



Ricardo Mario Arida

Abstract Neuroprotective and antiepileptogenic therapies have been extensively used for prevention and treatment of epilepsy. This chapter focuses on the positive influence of physical exercise observed in clinical studies and experimental models of epilepsy. We first give an overview of exercise in the healthy brain and in neurological diseases. We address the impact of previous exercise to reduce brain susceptibility to seizures after epilepsy has been established. Next, we explore the neurobiological mechanisms of these beneficial effects. Particular attention is given to the risks and benefits of physical exercise and possible seizure-precipitating factors related to exercise. Finally, this review provides evidence of exercise reducing comorbidities from epilepsy, improving the quality of life of people with epilepsy, and providing general guidance concerning participation in exercise/sport activities for people with epilepsy. Based on evidence from scientific literature, physical or sport activities represent an intervention that should be integrated as a complementary non-pharmacological treatment of epilepsy.

Keywords Epilepsy · Seizure · Physical exercise · Quality of life · Complementary therapy

21.1 General View of the Influence of Physical Exercise in the Healthy Brain and in Neurological Diseases

In the last decades, a growing literature has highlighted the beneficial role of physical exercise on several aspects of brain function. In humans, regular exercise promotes many benefits such as positive mood changes (Wegner et al. 2014), improves learning and memory (Hillman et al. 2002; Colcombe and Kramer 2003) and better

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academic performance in school-age children (Sibley and Etnier 2003). Along with cognitive abilities observed in humans, findings from animal studies have demonstrated that exercise increases synaptic plasticity in the hippocampus, a central structure for learning and memory, which consequently induces many structural changes such as synaptogenesis, neurogenesis, neuronal size, dendritic complexity, and blood vessels (van Praag 2008; Lista and Sorrentino 2010). In this regard, regular exercise has been recognised as an effective strategy in neuroprotection and neurorehabilitation. Among many beneficial effects, exercise intervention has been related to brain resilience. The role of exercise in resilience includes its involvement from brain development to ageing and for some common neurological disorders such as Alzheimer's, Parkinson, Stroke and Epilepsy (Arida and Teixeira-Machado 2021). Physical exercise at earlier stages of brain development, both on pre- and post-natal brain development (Gomes da Silva and Arida 2015), may persist in offspring and can increase brain resilience in later life. For adult, middle-aged or aged population, physical exercise can slow cognitive decline and protect against the consequences of stressful events. In addition to the preventive effects, physical exercise can promote neurobiological benefits for brain pathology, attenuating the decline in Alzheimer's disease, reducing disability and non-motor symptoms in Parkinson's disease and improving cardiorespiratory fitness, mobility and cognitive function after stroke. While the literature is extensive on the beneficial influence of regular exercise in different conditions, understanding of their effects in epilepsy is less known. The focus of this chapter is to highlight the potential contribution of exercise to epilepsy.

21.2 Non-pharmacological Treatments for Epilepsy

Neuroprotective and antiepileptogenic approaches have been extensively explored for preventing and treating epilepsy. Despite most seizures can be controlled with antiseizure medications (ASMs), about a third of patients with epilepsy have refractory or pharmacoresistant epilepsy, that is, seizures not controlled by two or more appropriately chosen ASMs (Perucca and Tomson 2011; Laxer et al. 2014). The most important concern with ASMs for the majority of patients is adverse effects, resulting in disability, morbidity, and mortality (Akyüz et al. 2021). Other current treatment options are brain surgery, device therapies such as vagus nerve stimulation, deep brain stimulation, and ketogenic diet (Sirven 2003). In this line, non-pharmacological therapies, combined with conventional medicine, have often been used by people with epilepsy (PWE) (Schachter 2008; Ekstein and Schachter 2010). Among the complementary and alternative medicines used by PWE, acupuncture, botanical/herbals, chiropractic care, magnet therapy, prayers, stress management, and yoga are frequently utilised (Saxena and Nadkarni 2011; Kiess et al. 2016). However, over the past decade, increasing scientific literature has supported the beneficial influence of physical exercise programmes as an adjunctive treatment for epilepsy (Arida et al. 2010a, b, 2013; Allendorfer and Arida 2018; van

der Kop et al. 2019; Arida et al. 2021/2021). Among exercise benefits, impact on the adjustment to seizures, ability to work and social functioning, comorbidities related to epilepsy, and consequently on the quality of life have been reported in PWE.

21.3 Physical Fitness in PWE

Physical fitness is characterised by several components carried out by an individual related to health, particularly cardiorespiratory endurance, body composition, muscular strength, and flexibility (Caspersen et al. 1985). For a long time, evidence from the literature has shown that PWE lead a sedentary life and has poor physical fitness compared to the general population. These findings are confirmed by interventions that analyse cardiorespiratory evaluation in this population. In cross-sectional studies, lower cardiorespiratory fitness has been reported in PWE compared to control health subjects (Vancini et al. 2010; Volpato et al. 2017). However, physical exercise interventions have improved cardiorespiratory fitness in PWE (Bjørholt et al. 1990; Nakken et al. 1990). Indeed, earlier studies have already reported that in general, PWE can obtain the same benefits from exercise programmes as people without epilepsy, specifically, increased maximal oxygen uptake and work capacity, reduced heart rate on a submaximal work level, weight reduction and body fat (Nakken et al. 1990; Arida et al. 2008). In clinical practice, PWE are often cautioned against participation in most physical/sport activities due to concerns of injury or precipitation of seizure or lack of information (Nakken et al. 1990; Eriksen et al. 1994; Arida et al. 2008/2013). Nevertheless, this scenario has changed in the last decades. While PWE were often advised against participating in physical activity in the past, there is now an increasing consensus stimulating their participation in physical/sports activities. To this end, the International League Against Epilepsy (ILAE) Task Force on Sports and Epilepsy provided general guidance concerning the participation of PWE in sport activities (Capovilla et al. 2016).

21.4 Effect of Physical Exercise on Seizure Discharges in the EEG (Electroencephalogram)

Initial clinical studies have demonstrated reduced electroencephalographic alterations induced by physical exercise. A classic study by Gotze and collaborators in the 1960s showed a reduction in the epileptiform discharge of PWE submitted to physical effort and hyperventilation (Gotze et al. 1967). Similar findings were reported in subsequent clinical investigations. Exercise on a cycle ergometer decreased the number of EEG alterations in almost all adults (Horyd et al. 1981) or children with epilepsy (Esquivel et al. 1991; Nakken et al. 1997). More recent studies have reinforced the role of exercise in reducing EEG alterations. A reduction of epileptiform

discharges was observed between the rest state and physical effort (82%) and in the recovery period (74%) in individuals with temporal lobe epilepsy (Vancini et al. 2010) and during the recovery period (72%) in subjects with juvenile myoclonic epilepsy (de Lima et al. 2011).

Positive findings in human studies are supported by animal models of epilepsy. Using the pilocarpine model of epilepsy, which reproduces the main features of human temporal lobe epilepsy, aerobic physical training reduced the number of population spikes in different concentrations of extracellular potassium or bicuculline and partially restored long-term potentiation (LTP) impairment observed in epileptic animals (Arida et al. 2004). In the genetic animal model for absence epilepsy (WAG/Rij strain), the total number and mean total duration of spike-wave discharges were significantly decreased after a swimming exercise programme (Aygun et al. 2019).

21.5 Effects of Physical Exercise on Seizure Occurrence

Although clinical data of programmes of physical exercise are still limited in the literature, in general, they indicate beneficial effects in seizure occurrence. A decrease in seizure frequency was observed in women with intractable epilepsy submitted to 15 weeks of aerobic exercise (Eriksen et al. 1994) or 3 months of combined exercise, i.e., aerobic training, resistance training and stretching (Häfele et al. 2021). In other studies, no increase in seizure frequency was reported after 4 weeks (Nakken et al. 1990) or 12 weeks of physical training (McAuley et al. 2001). Some factors that can interfere in the interpretation of clinical data are whether subjects included in the studies have a low or high number of seizures per week/month, are seizure-free at baseline, have different types of epilepsy and are physically active or sedentary subjects.

The protective role of exercise against seizures has also been evidenced in epidemiological studies. An analysis of the exercise habits of PWE showed that more than half of them never presented a seizure during exercise, and only 2% of them had exercise-induced seizures (Nakken 1999). In another investigation, only 5 of 250 individuals with epilepsy experienced a seizure during sport activities (Korczyński 1979). Similarly, Bjorholt et al. (1990) reported seizures in only 6 of 21 subjects, and no seizures were observed in another study (Steinhoff et al. 1996).

Remarkable, the majority of animal studies have reported the inhibitory effect of exercise on seizure occurrence. The first study that analysed the influence of an exercise programme in chronic animals with epilepsy showed an approximate reduction of 50% in the number of seizures during the training period (Arida et al. 1999). Subsequent investigations carried out by the same research group, using the same exercise protocol and animal model of epilepsy (pilocarpine model), confirmed the positive role of exercise in reducing seizure occurrence (Arida et al. 2003/2004/2007). These beneficial effects have also been confirmed in female

animals, i.e., exercise in a voluntary wheel running for 30 days reduced the seizure frequency in female rats (Vannucci Campos et al. 2017).

21.6 Antiepileptogenic Effects of Exercise

In humans, the impact of regular exercise for preventing epilepsy is still scarce. A population-based cohort study consisting of about 1.2 million men who were followed for a long period (up to 40 years) by a Swedish group revealed that low cardiovascular fitness early in life was associated with an increased risk of epilepsy later in life (Nyberg et al. 2013). The incidence of epilepsy over 20 years in a large number of participants of a long-distance Swedish cross-country ski race before retirement was up to 40–50% lower than their match controls (Ahl et al. 2019). It was also observed that ski racers with better performance (faster ski races) exhibited a lower incidence of epilepsy compared to those with inferior performance.

Emerging data in the literature using animal models reinforce human findings. The first evidence in the literature that examined the antiepileptogenic effect of exercise showed that an aerobic exercise programme retarded amygdala kindling development in rats (Arida et al. 1998). In this animal model, the administration of a subconvulsive electrical or chemical stimulus into a limbic structure such as the amygdala, hippocampus, entorhinal cortex or other brain areas leads to progressive and permanent amplification of seizure activity, culminating in generalised seizures. The number of electrical stimulations required to reach stage 5 (characterised by convulsive generalised seizures) was higher for the exercised animals compared to their controls. Motor symptoms such as frequency and intensity of seizures and *status epilepticus* duration induced by pilocarpine injection were attenuated in trained animals (Setkowicz and Mazur 2006). Posterior studies reinforced the role of previous physical exercise (with different paradigms such as swimming training and voluntary wheel running) to reduce seizure susceptibility induced by different chemoconvulsants such as homocysteine thiolactone, pentylenetetrazole, penicillin or kainite (Rambo et al. 2009; Souza et al. 2009; Reiss et al. 2009; Tutkun et al. 2010; Gomes da Silva et al. 2011; Kim et al. 2013/2014; Hrnčić et al. 2014; Holmes et al. 2015; Kayacan et al. 2016a/2016b/2019). Beneficial effects of exercise on behavioural findings included increased latency for the first motor signs and for reaching *status epilepticus*, lower intensity of motor signs, reduced seizure incidence and the number of animals that developed *status epilepticus*. Interestingly, most of these beneficial effects reported in the literature have been observed in male animals. In this regard, a study that verified the impact of exercise on seizure susceptibility in female rats showed an increase in latency to *status epilepticus* development after 30 days of voluntary exercise (Vannucci Campos et al. 2017). A systematic review and meta-analysis that addressed the impact of previous physical exercise programmes on seizure susceptibility in different animal models suggest a beneficial role in increasing resilience to developing epilepsy (Arida et al. 2021).

Exercise also plays an important role in influencing neurodevelopment and preparing the adult brain for life's challenges. An aerobic exercise programme in rats submitted during adolescence, i.e., postnatal day 21 (P21) to P60, delayed the onset and decreased the intensity of seizure motor symptoms after pilocarpine was administered at P150 (Gomes da Silva et al. 2011). Therefore, it can be suggested that the practise of physical exercise at earlier ages reduces the future risk of developing epilepsy.

21.7 Neurobiological Mechanisms by Which Exercise Can Reduce Seizures

In the epileptic condition, we can only make suppositions that might explain the beneficial effects of exercise in humans. Some possible mechanisms are the regulation of altered brain metabolism, neurotransmitter systems and growth factors. Among the neurotrophic factors, brain-derived neurotrophic factor (BDNF) plays an important role in neuroplasticity. However, in humans, BDNF blood levels detected in PWE are still inconsistent. Other possible mechanisms are antioxidant and anti-inflammatory effects (Radak et al. 2016) and reduced production of stress markers (Bouzd et al. 2014), which can confer neuroprotection. Stress is among the most frequently self-reported precipitants of seizures in PWE (McKee and Privitera 2017), and the sensitivity to stress is minimised after regular exercise (Salmon 2001). The mechanism by which physical stress might inhibit seizures has been previously postulated (Arida et al. 2009a). Since regular exercise has been known to attenuate excitotoxicity, neuroinflammation and oxidative stress, it can be suggested that physical exercise might modulate these effects, therefore, reducing seizure vulnerability.

In animal models, an extensive literature search has been conducted to understand the mechanisms of physical exercise in epilepsy. Brain structural and functional changes elicited by physical exercise have been suggested to mediate the inhibitory/excitatory balance to reduce seizure susceptibility. This section will describe the impact of exercise before and after epilepsy has been established.

21.7.1 Proposed Mechanisms of the Antiepileptogenic Effects of Exercise

Several studies have been conducted to verify whether physical activity influences the brain susceptibility to seizures or can prevent epilepsy development. The first evidence in the literature in animals that evaluated the antiepileptogenic effect of physical exercise demonstrated that an aerobic exercise programme retarded amygdala kindling development (Arida et al. 1998). As commented above, more stimulations were required in trained animals to reach stage 5 of kindling compared

with their controls. Neurotransmitter systems have been postulated to play an important role in these findings. The inhibitory effect of noradrenaline on kindling development is well documented. Previous studies have demonstrated that projections from the locus coeruleus to the hippocampal CA3/CA4 regions influence hippocampal excitability. For instance, noradrenaline depletion induced by DSP4, a noradrenergic neurotoxin, accelerates hippocampal kindling development (Bortolotto and Cavalheiro 1986). Administration of DSP-4 in the locus coeruleus in rats or lesions induced by dopamine beta-hydroxylase in knockout mice increased seizures elicited by chemoconvulsants (Szot et al. 1999; Giorgi et al. 2003). Thus, adrenaline administration before the first stimulus retarded the kindling development (Welsh and Gold 1984). It is well-established that physical exercise can increase the synthesis and release of catecholamines in several brain regions (Meeusen and De Meirleir 1995). It can be suggested that brain neurotransmitter alterations induced by exercise can mediate the inhibitory/excitatory balance to reduce seizure susceptibility and epileptogenesis. Changes in other neurochemicals, such as galanin, a neuropeptide that coexists with norepinephrine, are recognised to attenuate seizure susceptibility. Hippocampal injection of galanin reduces seizure activity, and galanin antagonists increase it. Accordingly, a treadmill exercise programme increased galanin mRNA levels in the locus coeruleus (O'Neal et al. 2001). In Reiss and collaborators' study, physical exercise reduced seizures induced by kainic acid, and a galanin antagonist reversed the positive effects of physical exercise (Reiss et al. 2009). Using other models of seizures, swimming exercise decreased epileptiform activity after penicillin microinjection into the somatomotor cortex (Tutkun et al. 2010) and spike amplitude and oxidative damage after intraperitoneal pentylenetetrazole administration (Souza et al. 2009) (for review see Arida et al. 2021).

21.7.2 Proposed Mechanisms of the Favourable Effects of Exercise in Chronic Epilepsy

Several investigations in animals have been undertaken to understand the basic mechanisms by which exercise can modify the natural course of epilepsy. It is well recognised that brain metabolism is altered during seizures and in the interictal period of epileptic seizures. Altered brain metabolism affords an indication of structures involved in the generation and propagation of epileptic activity. Using a quantitative [¹⁴C]2-deoxyglucose (2DG) method to measure the local cerebral metabolic rates for glucose (LCMRglu), a study analysed the altered functional activity in trained rats with epilepsy (Arida et al. 2003). Since the seizure occurrence is more frequent at rest and not during exercise, LCMRglu was inspected in the interictal period. Higher LCMRglu was observed in trained rats with epilepsy after 30 min of running on the treadmill. Thus, physical training normalised low metabolic rates in several brain areas in epileptic animals. Increased LCMRglu in the auditory and visual cortex was observed, regions involved in attention, vigilance and alertness (Vissing et al. 1996). The authors suggested that these specific brain changes are not

directly related to the exercise but to a higher mental alertness in exercising rats. As stated by Arida and collaborators (2009b), increased level of alertness and concentration is necessary during physical/sport activities, and the reduction of seizure susceptibility could be attributed to increased attention and vigilance during exercise.

Electrophysiological recordings in animals with epilepsy have demonstrated electrographic alterations in several limbic structures (Oliveira et al. 2011). However, how these electrophysiological changes can be altered by physical exercise in the epileptic condition has been poorly investigated. Studies have demonstrated that exercise can modify the electrophysiological properties of neurons. It is well-recognised that exercise increases hippocampal synaptic plasticity, including neurogenesis and hippocampal LTP. A classic study conducted at the end of the 1990s reported an increase in LTP amplitude in the dentate gyrus in slices from mice submitted to a voluntary running wheel (van Praag et al. 1999). Subsequent studies have reinforced these findings (Radahmadi et al. 2016; Dahlin et al. 2019). An “in vitro” hippocampal electrophysiological analysis monitored by extracellular field potentials recorded from the CA1 area in trained rats with epilepsy showed a reduction in hippocampal population spikes number in response to variable potassium and bicuculline extracellular concentrations (Arida et al. 2004). These findings indicate that physical training can modulate synaptic plasticity in rats with epilepsy, reducing seizure susceptibility.

During prolonged seizures, excitation of neurons can induce cell death resulting from increased oxidative stress, cytokine concentration and glutamate-induced excitotoxicity. Some mechanisms have been proposed to support the positive influence of exercise on the inhibitory/excitatory balance to reduce seizure activity (Arida et al. 2009b). Exercise promotes neurogenesis and survival of newborn neurons in the hippocampus (van Praag 2008) and can increase resistance to brain insults (Ang et al. 2003). Concerning the excitatory system, previous exercise can reduce hippocampal glutamate induced by kainic acid (Holmes et al., 2015). However, it must be determined whether this effect also occurs after epilepsy has been established. With reference to the inhibitory system, seizures induced by massive glutamate-induced calcium influx can lead to cell death after *status epilepticus*. Parvalbumin, a calcium-binding protein, mainly colocalised with the neurotransmitter GABA, displays a great affinity for calcium and may protect neuronal cells from calcium overflow (Sloviter 1989). A study that observed a reduced number of seizures in trained epileptic rats also reported an increased number of parvalbumin-positive cells and staining intensity of parvalbumin-fibres in the hilus of the dentate gyrus, suggesting a possible inhibitory effect of exercise in this condition (Arida et al. 2007).

Other possible neuroprotective factors not fully explored such as opioids, melatonin, neurotrophic factors, adenosine, stress and neurosteroids, may be considered. Based on previous investigations of opioid system involvement in seizure control (Hammers et al. 2007), it has been suggested that opioids released during exercise may inhibit seizures (Albrecht 1986). Indeed, alterations in central opioids after physical exercise have been investigated (Sforzo et al. 1986; Arida et al. 2015;

Varlinskaya et al. 2020). However, no studies have evaluated this effect during exercise in the epileptic condition. Although more research is needed to take conclusions on this topic, it can be suggested that physical exercise modulates the opioid system to reduce seizure susceptibility.

Human and animal studies have indicated the efficacy of melatonin as an anti-convulsant (Brigo and Igwe 2016). The seizure reduction has been observed in people with generalised epilepsy with generalised onset motor seizures after 8 weeks of melatonin treatment (Verma et al. 2021). The anticonvulsant role of melatonin has been demonstrated by using different convulsants including pentylenetetrazole, kainate, glutamate, and kindling (Banach et al. 2011). In the pilocarpine model of epilepsy, increased the number of spontaneous seizures was observed in pinealectomised rats, which was reverted by the administration of melatonin (de Lima et al. 2005). Thus, pinealectomy accelerated the amygdala kindling development in rats, indicating its endogenous neuroprotective effects. From the few studies that investigated the role of exercise in this condition, Silva de Lacerda and collaborators (2007) demonstrated that 30 days of aerobic exercise programme reverted the effects of pinealectomy on amygdala kindling development. However, it has been demonstrated that exercise can modulate plasma melatonin levels in humans (Carr et al. 1981; Escames et al. 2012); this effect in the brain with epilepsy needs to be confirmed.

Evidence for the involvement of neurotrophic factors in seizures and epilepsy has also been documented. BDNF is a member of the neurotrophic factor family strongly implicated in regulating the survival, growth and maintenance of neurons (Mattson et al. 2004). A large extent of literature has reported that under physiological conditions, exercise increases BDNF signalling. This increase is also observed following exercise to improve functional recovery after brain injury (Cotman et al. 2007; Di Raimondo et al. 2020). While studies have demonstrated that physical exercise increases BDNF expression in physiological and several pathological conditions, the precise role of BDNF in epilepsy is unclear. Some studies have shown that increased BDNF has epileptogenic action (Binder et al. 2001), whereas others have suggested that BDNF attenuates epilepsy (Simonato et al. 2006). The pro-epileptogenic effect was observed after the BDNF downregulation (Liu et al. 2013). Conversely, hippocampal BDNF administration retarded kindling development in rats (Osehobo et al. 1999). Some studies have analysed whether exercise alters BDNF expression following convulsant administration (Sartori et al. 2009; Lim et al. 2015); however, information concerning exercise in the chronic phase of epilepsy is limited. Two studies conducted by de Almeida and collaborators (2017/2018) demonstrated a beneficial role of the BDNF signalling pathway in epilepsy induced by exercise. An aerobic exercise programme in rats with epilepsy increased hippocampal BDNF expression, restored the overexpression its receptor TrkB to control levels, and modified the activation of intracellular signalling pathways associated with BDNF/TrkB (de Almeida et al. 2018). Using another exercise paradigm in rats with epilepsy, the resistance exercise restored to control levels the altered BDNF levels and ERK and mTOR activation (de Almeida et al. 2017).

Adenosine, another metabolic mechanism suggested as anticonvulsant has not yet been explored following exercise. Adenosine, a by-product of ATP, can be effective in inhibiting seizures. Increased metabolic activity induced by seizures yield increased extracellular adenosine severalfold (Beamer et al. 2021). Brain activation increases with the intensity of exercise, and intensive exercise preferentially uses lactate over glucose, increasing ATP production (Dworak et al. 2007), which suggests that adenosine can be a seizure-inhibitory component. However, further research must be conducted to evidence the effects of intensive exercise on adenosine and in the brain with epilepsy.

As mentioned above, although stress is a known precipitating factor for seizures, physical stress, that is, physical exercise, contributes to seizure reduction. Indeed, sensitivity to stress is decreased after regular physical/sport activities (Salmon 2001). Regular exercise modulates several neurotransmitter systems, reduces stress, decreases hypothalamic–pituitary–adrenal activity and adrenal glucocorticoids, which can decrease seizure susceptibility. Considering the beneficial role of regular exercise in stress reduction, neurosteroids have emerged as endogenous modulators of seizure susceptibility. Their action in reducing hypothalamic–pituitary–adrenal activity can adjust the homeostasis following stress, mainly by potentiating the activity of GABAA receptors (Reddy and Rogawski, 2002). Evidence from the research literature has suggested neurosteroids as a novel therapeutic strategy in epilepsy (Lévesque et al. 2020). For instance, experiments in rats showed that the infusion of finasteride, an inhibitor of the 5 α -reductase enzyme, used to inhibit neurosteroid synthesis exacerbated seizures (Lawrence et al. 2010). Although there is no evidence associating neurosteroids and exercise in the epilepsy condition, it has been postulated that exercise can induce neurosteroid release and act as an additional mechanism to reduce seizure susceptibility (Arida et al. 2010c). More information can be found in a review by Arida et al. (2009a).

21.8 Risks of Exercise in Terms of Inducing Seizures?

21.8.1 Seizure-Precipitating Factors

Although exercise has been shown to reduce epileptic activity and number of seizures, there are some factors that could induce seizures during exercise (Fig. 21.1). Among them are stress from competition, fatigue, hyperthermia, hypoxia, hyperventilation, hypoglycaemia and hyponatraemia. However, most of this information are largely speculative, except for some studies that demonstrate seizures induced by exercise. Some seizure events induced by exercise will be discussed in a subsequent section.



Fig. 21.1 Some speculative factors could cause seizures during exercise in adverse situations

Stress

As commented above, stress has been considered the most frequent precipitating factor reported by PWE. It has been suggested that the impact of stress on seizure induction is mediated by stress hormones (Maguire and Salpekar 2013). Stress can produce a dysregulation of hypothalamic–pituitary–adrenal axis, altering stress hormones and consequently may increase neuronal excitability. During competitive sports, psychological stress may lead to seizures. However, the literature has reported only a few cases in competitive sports (Bennett 1981) or after strenuous exercise (Ogunyemi et al. 1988).

Fatigue

Fatigue has also been considered a precipitating factor for seizures. It appears to be higher in PWE than in healthy subjects. It includes mental and/or physical tiredness, weakness, or exhaustion (Hernandez-Ronquillo et al. 2011). For a long time, the stress of competition has been cited to induce seizures in stress-sensitive subjects (Cordova 1993). Taking into account that athletic events such as marathon or triathlon can lead to both muscular and general fatigue, in the past, it was suggested that these exhausting activities might induce seizures through fatigue. However, no evidence in the scientific literature reports that seizures occur more frequently in exhaustive events than in other physical and/or sport activities.

Hyperthermia

Many studies have demonstrated that hyperthermia-induced seizures are the most common seizures during childhood. Extensive literature has demonstrated that in the developing rat brain, hyperthermia induces long-lasting changes in neuronal excitability (McClelland et al. 2011; Kasahara et al. 2019). As fever is the most common seizure trigger in infancy, it has been suggested that exercise at high temperatures and under extreme conditions of humidity increases the risk of hyperthermia in PWE. Hyperthermia is associated with pronounced reductions in cerebral blood flow. The hyperthermia-induced hyperventilatory response leads to a

reduction of arterial CO₂ pressure, triggering cerebral vasoconstriction and flow reduction. During prolonged exercise, hyperthermia is associated with an increased cerebral metabolic rate of oxygen and increased glucose utilisation (Nielsen and Nybo 2003). One proposed mechanism of seizure-induced hyperthermia is the reduced histamine level, and animal findings have indicated a possible anticonvulsant action of endogenous histamine (Yang et al. 2022). For instance, hyperthermia in rats decreases histamine blood levels, which increases seizure susceptibility (Gholipoor et al. 2013). In humans, exercise increases plasma histamine concentration (Anselme et al. 1994; Luttrell and Halliwill 2017). However, it is not established in the literature an association between hyperthermia induced by exercise and seizures. Only in rare cases can hyperthermia induce seizures in sport activities (Poussel et al. 2015) or in epileptic conditions such as Dravet syndrome (Verbeek et al. 2015).

Hypoxia

Hypoxia, another factor that may lead to neonatal seizures, has been clearly registered in different models of hypoxia and ischaemia (Sun et al. 2016). At high altitudes, many events such as hyperventilation, tachycardia and increased cerebral blood flow occur to maintain oxygen delivery to the brain. Thus, in hypoxia, increased signals from peripheral chemoreceptors lead to hyperventilation, resulting in respiratory alkalosis, which can consequently induce seizures. Hypoxia can occur in sports at high altitudes such as skiing or climbing. According to the ILAE task force for epilepsy and sports (Capovilla et al. 2016), these sport activities have been considered as moderate and major risk of injury/death should a seizure occur. Further information on general guidance concerning the participation of PWE in sport activities will be considered below.

Hyperventilation

Hyperventilation provokes absence seizures and is normally used in the clinic to diagnose absence seizures. In this regard, for a long time, it was believed that increased ventilation during exercise could trigger seizures. Voluntary hyperventilation causes a decrease in pCO₂, resulting in cerebral vasoconstriction, decreased cerebral blood flow and hypoxia. (Esquivel et al. 1991). However, the increased respiratory rate during physical exercise is a physiological response to increased metabolic demand. This compensatory homeostatic mechanism is different from the process of non-physiological hyperventilation, and therefore, the alkalosis induced by voluntary hyperventilation does not occur during exercise. In general, seizures are seldom triggered by physical activity.

Hypoglycaemia

Considering the association between metabolic disorders and epileptic seizures, it has been suggested that hypoglycaemia, produced by prolonged exercise, may also cause seizures. The brain is an organ with high metabolic demands. Insufficient energy in the brain can alter neuronal function (Fei et al. 2020), and decreased levels of glucose can increase cortical excitability in epilepsy patients (Badawy et al. 2013). However, seizures induced by hypoglycaemia are rarely cited in the literature. For instance, hypoglycaemic seizures are infrequent in PWE with adult-onset type 1 diabetes mellitus (O'Connell et al. 2008; Falip et al. 2014), and there is a low risk of seizure occurrence in patients with low blood glucose levels (Imad et al. 2015; Dudley et al. 2022).

Hyponatraemia

Electrolyte disturbances may affect the brain among many other organs, and its imbalance can cause seizures. Seizures are more frequently observed in patients with sodium disorders (especially hyponatraemia), hypocalcaemia, and hypomagnesaemia (Nardone et al. 2016). According to the ILAE, “a diagnosis of acute symptomatic seizure should be made in the presence of severe metabolic derangements (documented within 24 h by specific biochemical or haematologic abnormalities), drug or alcohol intoxication and withdrawal, or exposure to well-defined epileptogenic drugs” (Beghi et al. 2010).

Hyponatraemia associated with exercise is commonly reported in the literature and classified as a low blood sodium concentration (below 135 mmol/L) occurring during or following physical/sport activities (Hew-Butler et al. 2008). Hyperhydration commonly occurs during prolonged physical exercise such as marathon running and triathlon with an over-ingestion of isotonic or hypotonic liquids. To our knowledge, there is no information in the literature in PWE, and this association has been reported only in non-epileptic individuals (Kormann et al. 2012). In a prospective study of 26 marathon runners with hyponatraemia, three developed seizures (Davis et al. 2001).

21.8.2 Seizures Induced by Exercise

Although current literature has supported the protective role of exercise against seizures, the precipitation of seizures by exercise has also been documented. These comprise people with idiopathic generalised epilepsies (Ogunyemi et al. 1988; Schmitt et al. 1994; Werz 2005) and symptomatic focal epilepsies of the frontal (Simpson and Grossman 1989) and temporal lobe (Sturm et al. 2002) origin. Clinical cases have been reported, generally concerning stimulus-related or reflex epilepsy syndromes (Ogunyemi et al. 1988; Schmitt et al. 1994; Sturm et al. 2002). For

instance, Ogunyemi et al. (1988) reported three cases of exercise-induced seizures, and Korczyn (1979) described five cases of exercise-induced seizures, one of whom was a long-distance runner. In Sturm's study (2002), two cases of exercise-induced seizures were more likely during strenuous exertion. Therefore, the association between these factors and seizure occurrence is speculative.

21.9 Physical Exercise Minimising Comorbidities Associated with Epilepsy

PWE frequently experience medical and psychiatric comorbidities, which have a negative impact on quality of life. For most PWE, some comorbidities are more disabling than the seizures themselves (Ottman et al. 2011) and depression and anxiety are largely observed. Abnormalities of neurotransmitter systems are frequently found in mood disorders and epilepsy. As regular physical exercise can modulate several neurotransmitter systems, neurotrophic factors and hypothalamic–pituitary–adrenal activity (Meeusen and De Meirleir; Walsh and Tschakovsky 2018), it has been suggested that in the epileptic condition, exercise can reduce stress, seizure susceptibility and consequently epilepsy comorbidities. Indeed, findings from human studies have clearly reported that the level of depression in PWE is lower among those who exercise regularly (Roth et al. 1994; Nakken 1999; de Lima et al. 2013). Brief considerations of the influence of exercise on the modulation of the neurotransmitter systems to attenuate depression in the epileptic condition are reviewed elsewhere (Arida et al. 2012).

Cognitive impairment is a prominent element of depression. Cognitive deficits have been associated with the aetiology of epilepsy, recurrent seizures, use of ASMs or their association (Leeman-Markowski and Schachter 2016). In this respect, people with mesial temporal lobe epilepsy with high seizure frequency perform worse on tests of anterograde memory than those with low seizure frequency (Veltzenlogel et al. 2014). The impact of exercise under these conditions has been poorly examined. One study showed improvement of verbal memory following a physical exercise programme in PWE (Allendorfer et al. 2019). In another study, improvement in executive function, mainly in attention and language tasks was observed after 3 months of physical exercise in adults with epilepsy (Feter et al. 2020). Although positive effects of physical exercise have been reported on cognition, there is a lack of randomised controlled trials to confirm the beneficial effects of regular exercise in reducing cognitive impairment in PWE (Allendorfer and Arida 2018). Additionally, it has also been proposed an increased risk for cognitive impairment with the use of ASMs (Novak et al. 2022).

21.10 Physical Exercise and ASMs

Some ASMs have been associated with weight gain (Ben-Menachem 2007; Hamed 2015) and bone loss (Elliott and Jacobson 2006; Zhong et al. 2019). Exercise habits are important for preventing bone health. PWE are less likely to participate in physical/sports activities than the general population, supporting the idea that lifestyle influences weight. Although no clinical studies have examined whether regular exercise can prevent or minimise bone loss in the epileptic population, exercise should also be included as an osteoprotective behaviour. Whether physical exercise can alter ASMs concentration and trigger seizures is uncertain. The few clinical studies that analysed this issue have not reported significant changes in ASMs concentration following exercise (McAuley et al. 2001; Nakken et al. 1990). The metabolic changes of ASMs need to be evaluated in randomised controlled studies in the exercise condition. The findings of animal studies have demonstrated that exercise can improve the effectiveness of ASMs. In one study, while carbamazepine in doses of 25 and 50 mg/kg was not effective to reduce seizures induced by pentylenetetrazol, an associative treatment with exercise and carbamazepine in these doses was significantly positive in reducing seizures, suggesting the efficacy of exercise to reduce the ASMs dose (Barzroodi Pour et al. 2021). In another investigation, a physical exercise programme potentiated the topiramate antiepileptic activity to pentylenetetrazol-induced seizure (Soleimani Meigoni et al. 2021). These animals' studies indicate that exercise have a potential role as complementary treatment to reduce the ASMs as well as their side effects. For more information about this issue, see the recommendations from the ILAE task force on sports and epilepsy (Capovilla et al. 2016).

21.11 ILAE Task Force on Sports and Epilepsy

Negative views and inappropriate advice due to inadequate knowledge about physical exercise among health professionals have prevented PWE from participating in regular physical exercise. A low degree of participation in physical activities is due to overprotection, ignorance about the benefits and risks associated with these activities, or fear that exercise can induce seizures (Stanuszek et al. 2015). To this purpose, the ILAE Task Force on Sports and Epilepsy (ILAE, 2012. <https://www.ilae.org/about-ilae/public-policy-and-advocacy/epilepsy-and-sport-project-stand-up-for-epilepsy>) published a consensus paper to provide general guidance concerning participation in exercise/sport activities for PWE (Capovilla et al. 2016). Factors considered in this special report included “the type of sport, the likelihood of a seizure occurring, the type and severity of the seizures, seizure-precipitating factors and usual timing of seizure manifestation”. An analysis of which sport activities

might be indicated based on the above factors, led to classify sports into three categories based on the potential risk of injury or death should a seizure occur: “Group 1, sports with no significant additional risk; group 2, sports with moderate risk to PWE, but no risk to bystanders; and group 3, sports with a major risk for PWE and risks for bystanders”. This analysis took into account different conditions including “people who had one or more acute symptomatic seizures; people who had a single, unprovoked seizure; people who are seizure-free; people with sleep-related seizures only; people continuing to have seizures without impaired awareness; people continuing to have seizures with impaired awareness; people in whom epilepsy has resolved and medication withdrawn”. Some of these recommendations may be applied at neurologist’s judgement.

21.12 Final Considerations

An increasing number of investigations have reported the effectiveness of physical exercise, both reducing seizures and improving the quality of life of PWE. This issue has been addressed in clinical and non-clinical research. Although there are some factors that could trigger seizures during exercise, seizures induced by exercise is uncommon. Findings from animal studies have pointed out several mechanisms to reduce seizure susceptibility, including modulation of neurotransmitters, the opioid, inflammatory and adenosinergic systems, neurotrophins, brain metabolism and oxidative stress, hypothalamo-pituitary-adrenocortical axis and steroid hormones. Although the positive effects of exercise before and after epilepsy in preclinical studies are clearly evidenced in the literature, it is unclear whether exercise during this latent period, after the brain insult but before spontaneous seizures, can prevent or minimise the progression of disease. Physical/sport activities should also be introduced in humans to attest to other beneficial health aspects of exercise.

While experimental studies have provided substantial information on the positive effects of exercise on epilepsy, evidence from human studies is still limited. Clinical studies applying a physical exercise programme, in general, have reported a beneficial impact on seizure frequency and several factors related to the quality of life. Indeed, the importance of exercise in the epileptic condition goes beyond reducing the seizure frequency or seizure susceptibility (see Fig. 21.2). Improvements in cognitive, emotional and behavioural components, social functioning, family stability and self-esteem are decisive to the quality of life of PWE (Devinsky 1996; Jacoby and Baker 2008). Well-controlled longitudinal studies must establish greater clinical evidence. Overall, physical or sport activities is an intervention that should be integrated into the current health care system for preventing and as a complementary non-pharmacological treatment of epilepsy.

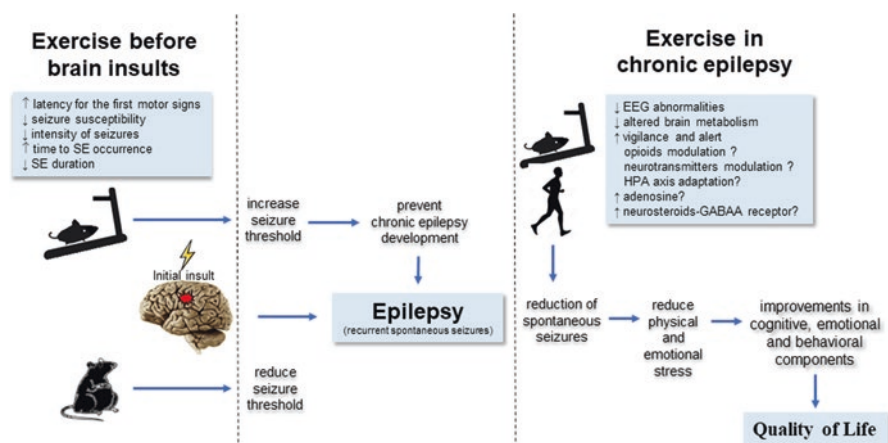


Fig. 21.2 Impact of previous exercise to reduce seizure susceptibility and in chronic epilepsy to reduce suppress spontaneous seizures and quality of people with the epilepsy

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Chapter 22

Ketogenic Diet and Drug-Resistant Epilepsy



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Abstract The ketogenic diet is a high-fat, low-carbohydrate, and restricted protein diet that is used in children with drug-resistant epilepsy. Over the past years, there has been a focus on the use of ketogenic dietary therapy in different types of epilepsy and epilepsy syndromes as well as etiologies and as an alternative treatment in refractory and super refractory status epilepticus. Ketogenic dietary therapies probably have multiple mechanisms of action that may work synergistically. It has been shown that children with Angelman syndrome, Dravet syndrome, febrile infection-related epilepsy syndrome, infantile epileptic spasms, epilepsy with myoclonic-atonic seizures, and tuberous sclerosis complex respond particularly well to the diet. In many patients, the diet improves not only the epilepsy but also cognition and behavior. Current evidence shows that ketogenic dietary therapies should be used earlier in the treatment algorithm for several syndromes.

Keywords Ketogenic dietary therapy · Epilepsy · Drug-resistant · Epilepsy syndromes · Etiology

22.1 Introduction

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and restricted protein diet that is used in children with drug-resistant epilepsy. It was originally developed in the 1920s but fell into disuse with the advent of the first antiseizure medications (ASMs). Nevertheless, in spite of these drugs, around one-third of patients with epilepsy remained drug-resistant. A search for alternative treatment options resulted in renewed interest in the KD in the 1990s.

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Many studies, including randomized controlled trials, of ketogenic dietary therapy (KDT) for people with drug-resistant epilepsy have been conducted since then (Martin-McGill et al. 2020). In 2009, the first international expert consensus on the clinical management and indications of the diet was published (Kossoff et al. 2009), followed by an extended and updated version in 2018 (Kossoff et al. 2018). KDTs are currently considered an evidence-based treatment for drug-resistant epilepsy.

Over the past years, there has been a focus on the use of KDT in different types of epilepsy and syndromes, as well as etiologies and as an alternative treatment for refractory (RSE) and super refractory status epilepticus (SRSE).

Children on the diet have been shown to have a 40–50% chance of at least a 50% seizure reduction (Kossoff et al. 2018). A Cochrane review found that in children, seizure freedom rates on the classic 4:1 KD ranged from 10% to 55%, while the rates of seizure reduction ranged from 55% to 85% (Martin-McGill et al. 2020). KDT is also considered to have a long-term impact on cognitive function and behavior, attention (Pulsifer et al. 2001), quality of sleep (Hallböök et al. 2007), and reduced costs (de Kinderen et al. 2015), although controlled randomized trials to evaluate these benefits are still lacking.

22.2 Ketogenic Dietary Therapies

The classic KD is a rigid, mathematically calculated, personalized, and medically controlled diet. The 4:1 ketogenic ratio is the most commonly used ratio and corresponds to 4 g of fat to 1 g of protein plus carbohydrates, representing 90% of calories from fat. New variants with fewer restrictions and lower fat content, including the modified Atkins diet (MAD), the medium-chain triglyceride diet (MCT), and the low glycemic index treatment (LGIT), were developed to make the diet less complicated and more palatable, especially for older children and adults (Caraballo and Armeno 2023).

A randomized clinical trial of 158 children with drug-resistant epilepsy (Sondhi et al. 2020) found that the median reduction in seizure burden was similar between the KD, MAD, and LGIT. Median seizure reductions were 66% for the KD, 45% for the MAD, and 54% for the LGIT diet, with fewer adverse events found when using the latter.

22.3 Mechanisms of Action

How exactly KDTs work is still not well understood, although there are different hypotheses about its mechanism of action. KDTs probably have multiple mechanisms of action that may work synergistically.

KDTs affect the brain metabolism essentially by “tricking” the brain into believing it is in a fasting state. The body is deprived of carbohydrates, given sufficient protein, and large amounts of fat. The body quickly depletes carbohydrates and begins to use fat as a primary source of energy.

The first mechanism of action of the diet is often associated with metabolic changes that cause increased levels of ketone bodies. These, produced by ketogenesis in the mitochondria of liver cells, can cross the blood-brain barrier and enter the brain. The three types of ketone bodies are acetone, acetoacetate, and beta-hydroxybutyric acid (BHB). Different studies suggest that the antiepileptogenic effect is due to the fact that ketone bodies change the ability of neurons to cause excitatory mechanisms or that they change the activity of the mitochondria, the so-called “power center” of the cell (Rogawski et al. 2016).

Ketone bodies are a more efficient energy source than glucose, metabolize faster, and can bypass the glycolytic pathway. The glycolytic restriction is considered to be another important mechanism mediating the antiseizure properties of the ketogenic diet (Rogawski et al. 2016). By the inhibition of glycolysis and fatty acids, KDT increases mitochondrial oxidation-induced ATP generation and decreases oxidative stress, the latter of which plays an important role in epileptogenesis (Zhu et al. 2022).

Another mechanism of action of the diet may be through increases in the levels and types of polyunsaturated fatty acids (PUFAs) in the blood circulation, liver, and brain. The effect of PUFAs on ion channels, including voltage-gated sodium and potassium channels, may lead to seizure reduction (Rogawski et al. 2016).

The diet seems to cause changes in the amino acid metabolism in the brain, especially glutamic acid. Ketosis could stimulate the conversion of glutamate to glutamine, leading, on the one hand, to a more efficient removal of glutamate, the main excitatory neurotransmitter, and, on the other hand, to a more efficient conversion of glutamine to gamma-aminobutyric acid (GABA), the most important inhibitory neurotransmitter. An increase in GABA activity generates an inhibitory effect that decreases susceptibility to epileptic seizures (Yudkoff et al. 2008).

In addition, BHB is a very effective inhibitor of glutamate transport (VGLUT2) and increases GABA synthesis (Newman and Verdin 2017). When ketogenesis is activated, such as during fasting, blood levels of BHB have been shown to rise faster than either acetoacetate or acetone (Lincoln et al. 1987). Interestingly, acetoacetate, BHB, and pyruvate inhibit Cl^- -dependent glutamate uptake, with kinetics indicating competition for the Cl^- -binding site. All these effects require the anions to be exposed to the extravesicular side of the membrane transporter. In addition, studies of isotopically labeled BHB show that it is used as a substrate for the synthesis of glutamine and other amino acids (Yudkoff et al. 2001). In agreement with these observations, data from clinical studies of children with drug-resistant epilepsy on KDT for epilepsy showed that cerebrospinal fluid GABA levels are higher on KDT, and the highest levels correlate with best seizure control (Dahlin et al. 2005).

The low-glucose and -insulin conditions caused by KDTs may reduce the activity of the mechanistic target of the rapamycin complex 1 (mTORC1) signaling pathway, which plays a role in the development of malformations of cortical development,

such as focal cortical dysplasia (FCD) or hemimegalencephaly (McDaniel et al. 2011). This property of KDTs to decrease mTOR activity may be of great value for epilepsies considered mTOR-pathies, particularly tuberous sclerosis complex (TSC) where mutations affect the gene products hamartin and tuberin resulting in a loss of inhibitory control on mTOR.

In addition, changes in the gut microbiome on KDT have been reported with a decrease in pro-inflammatory and an increase in beneficial bacteria possibly playing a role in the antiseizure effect (Olson et al. 2018; Zhu et al. 2022).

Recently it has been suggested that increased nicotinamide adenine dinucleotide (NAD+) may be a common mechanism underlying the beneficial effects of ketogenic diet therapy.

Finally, another potential mechanism is that of sebacic acid (SA), which is an inhibitor of P-glycoprotein activity, and reduced the drug-resistant phenotype in an experimental epilepsy model (Enrique et al. 2021). Interestingly, SA is a metabolic product (via ω -oxidation) of decanoic acid, one of the main components of the medium-chain triglyceride KD that is raised in the plasma of patients intaking such a diet (Hughes et al. 2014).

Probably for some epilepsies a certain mechanism is important, while for others another mechanism mediates the antiepileptogenic effect. For example, it is assumed that for infantile spasms, ketosis plays an important role, whereas for Doose syndrome or epilepsy with myoclonic-atonic seizures (EMAS) calorie restriction may be the mechanism of action. Knowledge of the means by which diet exerts a positive effect on seizure control in relation to the epileptic syndrome may in the future make it possible to modify the characteristics of the diet and optimize its use (Kossoff et al. 2016) (Table 22.1).

Table 22.1 Mechanisms of action of ketogenic dietary therapies

Direct action of the ketone bodies (Huttenlocher 1976; Gasior et al. 2006)
Acidosis (Huttenlocher 1976; Ross et al. 1985)
Cellular y extracellular dehydration (Chesney et al. 1999)
Hypoglycemia (Ross et al. 1985; Fraser et al. 2003)
Neuronal firing (Bought et al. 2000)
Synaptic function (Szot et al. 2004)
Neurotransmitters (Yudkoff et al. 2001; Weinshenker 2008)
Effect of lipids on neuronal excitability (Likhodii et al. 2003)
Source of energy in the brain (Kumar et al. 2016)
Alteration of the mitochondrial metabolism (Sullivan et al. 2004)
Ion channels (Zhu et al. 2022)
mTOR pathway (McDaniel et al. 2011)
Changes in the gut microbiota (Olson et al. 2018)
Multiple effects of nicotinamide adenine dinucleotide (Elamin et al. 2020)

22.4 Indications: Inclusion and Exclusion Criteria

Although traditionally adolescents and adults were considered not to be candidates for KD treatment because of the unpalatability and strictness of the diet, over the past years there has been increased interest in the use of the diet in these age groups and research suggests similar outcomes as in children (Liu et al. 2018). The different diet options, such as the MAD, the LGIT, and formulas, facilitate the use of KDTs in different age groups and different epilepsy syndromes.

KDT showed to be effective in the majority of infants with treatment-resistant epilepsy (62.4%) (Armeno et al. 2021). Since the first study on KDT in infants by Nordli et al. (2001), several studies have reported similar findings (Kang et al. 2005; Dressler et al. 2015; Wirrell et al. 2018). In a meta-analysis and systematic review including a total of 534 infants with efficacy data, approximately 60% achieved more than 50% seizure reduction of whom 33% became seizure-free (Lyons et al. 2020). Currently, KDT is also used in very young infants. Recently, our group has treated nine patients with developmental and epileptic encephalopathies (DEE) burst-suppression with drug-resistant seizures of onset in early infancy initiating the diet between 1 and 3 months of life. A more than 50% seizure reduction was observed in six of them (unpublished data).

For two neurometabolic diseases, glucose transporter 1 deficiency syndrome (Glut1DS) and pyruvate dehydrogenase deficiency (PDHD), KDTs are the first-line treatment. In addition, currently, it is considered that KDTs may be offered earlier in the treatment scheme for Angelman syndrome, Dravet syndrome (DS), febrile infection-related epilepsy syndrome (FIRES), infantile epileptic spasms (IES), epilepsy with myoclonic-atonic seizures (EMAS), and TSC. Children with gastrostomy tubes are also candidates for earlier consideration of the treatment (Kossoff et al. 2018).

Importantly, KDTs are absolutely contraindicated in several disorders of fat metabolism because of the high amounts of lipids in the diet (Table 22.2). Before starting the diet, a child should be screened for disorders of fatty acid transport and oxidation, especially when the etiology of the epilepsy is not known (Kossoff et al. 2018).

22.5 Ketogenic Dietary Therapy in Epileptic Syndromes

KDTs are particularly useful in certain epilepsy syndromes (Nangia et al. 2012). In our multicenter study assessing the efficacy and the tolerability of the classic KD for different epilepsy syndromes in 216 patients, 140 (65%) patients remained on the diet 18 months after KD initiation. Of these patients, 31/140 (22%) became seizure-free and 79/140 (56%) had a greater than 75% seizure reduction. The KD was found to be most effective in patients with EMAS, Lennox-Gastaut syndrome (LGS), IES,

Table 22.2 Inclusion and exclusion criteria for ketogenic diet therapy

Inclusion criteria	Exclusion criteria
Patients with drug-resistant epilepsies	Contraindications to the use of KDT (absolute)
Age: preferably between 1 and 8 years (newborns, infants, children, adolescents, and adults)	Primary carnitine deficiency
GLUT 1 deficiency syndrome	Carnitine palmitoyltransferase I or II deficiency
Pyruvate dehydrogenase deficiency	Carnitine translocase deficiency
Phosphofructokinase deficiency, type V	Beta-oxidation defects: long, short, and medium chain acyl dehydrogenase deficiency and long and medium chain 3-hydroxy acyl CoA deficiency
glycogenosis, and mitochondrial respiratory chain disorders	Pyruvate carboxylase deficiency
Structural neurological damage does not contraindicate the use of the ketogenic diet	Porphyria
IQ: patients are included regardless of IQ	Other exclusion criteria
	Patients with liver, kidney, heart, gastrointestinal, and psychiatric disease
	Family history of risk to strictly maintain the diet

and DS, and patients with structural focal epilepsy secondary to malformations of cortical development and TSC. In addition, children with FIRES and with developmental and epileptic encephalopathy with spike-wave activity in sleep (D/EE-SWAS) responded well to the diet (Caraballo et al. 2011).

The first series on the efficacy of KDT in DS (Caraballo et al. 2005) described a group of 42 patients of whom 75% had a more than 50% decrease in seizure frequency. Further studies (Nabbout et al. 2011; Laux and Blackfort 2013; Dressler et al. 2015) showed similar results. A prospective study by Yan et al. (2018) evaluating 20 DS patients with *SCN1A* mutations receiving KDT found a seizure reduction of 85% and an improvement in cognition of 80%. Currently, KDTs are the second-line treatment for DS as outlined by the North American consensus panel (Wirrell et al. 2017; Cross et al. 2019).

Patients with EMAS have been shown to respond particularly well to the diet. Oguni et al. (2002) analyzed 81 patients with EMAS and considered that KDT was the most effective treatment for EMAS, before ACTH and ethosuximide. In a prospective cohort of 11 patients with EMAS treated with KDT (Caraballo et al. 2006), it was found that half the children showed a more than 50% reduction in seizures while 18% became seizure free. In another series of children with EMAS treated between 1998 and 2005, treatment with KDT was associated with the highest seizure freedom rate (Kilaru and Bergqvist 2007).

Nordli et al. (2001) reported the first retrospective series of infants with treatment-resistant epilepsy treated with KDT and observed that those with IES had a specifically good response rate. Three prospective studies in children with IES on KDT showed a median response rate of 65% and a median seizure-free rate of 27.8% (Hong et al. 2010; Lee et al. 2013; Pires et al. 2013). In addition, of three children with infantile spasms without hypsarrhythmia that were placed on KDT, two responded well to the diet (Caraballo et al. 2011). The 2010 U.S. consensus report of the Infantile Spasms Working Group considered KDT a second-line therapy if

ACTH and vigabatrin (VGB) fail or are not indicated in a given patient (Pellock et al. 2010). In a randomized controlled trial (RCT) of infants allocated either to KDT or high-dose ACTH it was found that KDT is as effective as ACTH in the long term but is better tolerated. The authors recommend using ACTH as the first choice to achieve short-term remission if previous VGB treatment is not given. However, with prior VGB, they found that KDT was at least as effective as ACTH in the short term and was associated with lower relapse rates in the long term. They concluded that KDT is an appropriate second-line treatment after VGB (Dressler et al. 2019). Our group found very good results in 30 infants with IES treated with KDT (Armeno et al. 2021).

A retrospective study of 71 children with LGS treated with the diet found that approximately half of the children responded at 12 months (Lemmon et al. 2012). In a series of patients with LGS, of whom 20 were placed on the classic KD, the diet was found to be effective and well tolerated not only for those with an unknown etiology but also for those with structural LGS (Caraballo et al. 2014). In another study using the MAD to treat children with LGS, adverse effects were mild and the diet was found to be effective and well-tolerated (Sharma et al. 2015).

Of three patients with drug-resistant epilepsy of infancy with migrating focal seizures (EIMFS) with onset before 5 months of age treated with the classic KD, one became seizure free with significant improvement of neurocognitive function, the other had a 75–99% seizure reduction with moderate psychomotor improvement, while the remaining patient had a less than 50% seizure reduction. In all three patients, tolerability was good (Caraballo et al. 2015).

DEE with spike-wave activity in sleep (DEE-SWAS) is often drug-resistant and some patients develop steroid dependency. In a study of 65 children with DEE-SWAS, 12 were placed on the KD as an add-on to the use of one to three ASMs. The KD was effective regardless of etiology suggesting that the diet is a good treatment option for patients with DEE-SWAS, not only for structural cases but also for those with an unknown etiology (Reyes et al. 2015). In another series of 42 patients receiving oral steroids combined with the KD, 13 had DEE-SWAS. Eight of these patients responded well to the diet, and the authors concluded that patients with steroid-dependent DEE-SWAS seemed to be the best candidates for the diet (Ville et al. 2015).

In a retrospective study of a historic cohort of patients with Aicardi syndrome treated with KDT since 1994, 15 patients, aged 4 months to 34 years, were analyzed. Ten (67%) patients experienced a $\geq 50\%$ seizure reduction after 3 months, with three (20%) having a $\geq 90\%$ reduction. Only one patient was seizure free for a short period of time. The authors considered KDT to be helpful in Aicardi syndrome, especially in patients who were more drug resistant and did not have IES at KDT initiation (Sanchez et al. 2021).

Although there is still a lack of randomized controlled trials, the results of these studies support the concept that the KD may be introduced earlier in the treatment algorithm of many syndromes (Table 22.3).

Table 22.3 Epileptic syndromes and types of epilepsy that respond well to ketogenic dietary therapy

Epileptic syndromes and types of epilepsy	
Early-onset epileptic encephalopathies with burst suppression	Febrile infection-related epilepsy syndrome (FIRES)
Infantile epileptic spasms (IES)	Rasmussen's syndrome
Dravet syndrome (DS)	Focal structural epilepsies or secondary to metabolic diseases
Epilepsy of infancy with migrating focal seizures (EIMFS)	Focal epilepsies of unknown cause
Epilepsy with myoclonic-atonic seizures (EMAS)	Juvenile myoclonic epilepsy (JME)
Lennox-Gastaut syndrome (LGS)	Childhood epilepsy with myoclonic absences
Landau-Kleffner syndrome (LKS)	Other drug-resistant childhood absence epilepsies
Developmental and epileptic encephalopathy with spike-wave activity in sleep (DEE-SWAS)	
Myoclonic status in non-progressive encephalopathies	
Developmental and epileptic encephalopathies (DEE) with specific or nonspecific etiologies	

22.6 Ketogenic Dietary Therapies and Etiology

Determining the etiology of the different epileptic syndromes and/or types of epilepsy is crucial in the evaluation of treatment options, including KDT. These causes may be genetic, metabolic, autoimmune, infectious, as well as structural, both congenital and acquired. Although based on small, uncontrolled studies, evidence is increasing that the response of drug-resistant epilepsies to KDT may be related to the underlying etiologies.

Angelman syndrome is a genetic neurodevelopmental disorder that is associated with epilepsy in 80% of patients. In a series of patients with Angelman syndrome, the KD was found to be successful (Thibert et al. 2009). Subsequently, different studies reported the successful use of the LGIT in patients with Angelman syndrome (Thibert et al. 2012; Grocott et al. 2017).

Previously, different studies on the efficacy of KDT according to etiology reported good results in small series of children with Rett syndrome (Liebhaber et al. 2003; Giampietro et al. 2006) and Lafora disease (Cardinali et al. 2006).

Different studies have shown that KDT is a good option for children with TSC (Kossoff et al. 2005, 2007; Coppola et al. 2006; Martinez et al. 2007; Park et al. 2017; Fang et al. 2022). In addition, it has been suggested that the use of KDTs for more than 2 years may be beneficial for patients with TSC who have achieved seizure freedom (Park et al. 2017). In patients with psychomotor disabilities, cognitive and behavioral improvements were observed (Fang et al. 2022).

There is a growing interest in genetic diagnosis in epileptic encephalopathies, as genetic etiologies are often associated with developmental delay in addition to the seizure burden. Among others, ion channel genes have been demonstrated to be implicated in severe epileptic encephalopathies.

DS is associated with mutations in the sodium channel $\alpha 1$ -subunit gene (*SCN1A*) in 70–80% of individuals. As described above, children with DS have been shown to respond particularly well to the diet.

A retrospective study evaluating the efficacy of KDT in 155 patients with DEE associated with specific gene mutations found that patients with *SCN2A*, *STXBPI*, *KCNQ2*, and *SCN1A* mutations had a better response to the KD, while those with *CDKL5* mutations showed a worse response (Ko et al. 2018). These findings are consistent with those of previous studies in patients with EE with *SCN1A* and *SCN2A* mutations. Patients with Ohtahara syndrome and *KCNQ2* or *STXBPI* mutations responded particularly well to the diet (Ko et al. 2018). On the other hand, another study evaluating the efficacy of KDT for IS in patients with and without different causative genetic mutations showed that patients with *CDKL5* mutations had a significantly better response to KDT (87.50%) than patients without *CDKL5* mutations ($p = 0.03$) (Wang et al. 2022).

As mentioned above, inhibition of the mTOR pathway is one of the proposed mechanisms of action of KDT. In a study of 25 patients with pathologically confirmed FCD treated with KDT, 12 had germline or somatic detectable mTOR pathway mutations. The authors found that after 3 months the efficacy of KDT was superior in patients with detectable mTOR pathway mutations than in those without detectable mTOR pathway mutations, although the difference was not statistically significant (responder rates of 58.3% vs. 38.5%, $p = 0.434$) (Ko et al. 2022).

In a study comparing the outcome of KDT according to the different etiologies, a better response was found in patients with a genetic and unknown etiology. The authors hypothesize that as the effectiveness of KDT was comparable in the unknown and the genetic etiology, many unknown etiologies may represent yet-unidentified genetic causes (Breu et al. 2021). This highlights the importance of identifying genetic etiologies and determining if they are candidates for KDT treatment.

KDT is also an option for patients with different inherited metabolic disorders (Caraballo 2017). As said above, in GLUT-1DS and PDHD the diet is the treatment of choice. Since first described in 1991, more than 300 patients with GLUT-1 deficiency syndrome have been reported (De Vivo et al. 1991). Recently, MAD was also shown to be effective in this group of patients (Ito et al. 2011). Several reports have suggested the use of KDTs in other metabolic disorders, mainly those of intermediary metabolism (glycogen storage diseases) (Busch et al. 2005; Brambilla et al. 2014) and disorders of mitochondrial energy supply, as the diet may cause specific changes in mitochondrial metabolism or function.

Clinical findings have indicated that the KD may control seizures in children with drug-resistant epilepsy associated with mitochondrial respiratory chain complex defects (Kang et al. 2007; Lee et al. 2008), mitochondrial DNA depletion syndromes, such as Alpers-Huttenlocher syndrome (Khan et al. 2012), and disorders of mitochondrial transcription and translation (MELAS) (Steriade et al. 2014).

Early myoclonic encephalopathy (EME) is a syndrome with a poor prognosis and no effective therapy (Caraballo 2018). Three cases of neonatal nonketotic hypoglycemia and EME were reported (Cusmai et al. 2012). The KD in

combination with ASMs led to a dramatic seizure reduction and improvement in quality of life.

KDTs are also considered in inflammatory as well infectious etiologies regardless of the types of seizures and epilepsy or epileptic syndrome. In patients without a history of seizures who develop seizures that progress over a few days to SE, and in whom tumor, stroke, intoxication, vasculitis, and metabolic, infectious, or structural causes were ruled out, new-onset RSE (NORSE) should be suspected (Hirsch et al. 2018). This is an important concept as it allows us to take the diet into consideration as an add-on treatment option in an early stage of the disease. FIRES is a subtype of NORSE that requires a febrile infection that started between 2 weeks and 24 h prior to the onset of RSE, with or without fever at the onset of the status epilepticus (SE) (Hirsch et al. 2018). The first report on the use of the diet in FIRES was by Nabbout et al. (Nabbout et al. 2010). The authors evaluated nine patients with FIRES and drug-resistant SE seen over 12 years. The KD worked in seven. In our series (Caraballo et al. 2013), two of 12 children with FIRES with a mean follow-up of 6.5 years were put on KDT in the acute phase; one had a 50–75% seizure reduction, while the other had a seizure reduction of less than 50%. Good efficacy and improved cognitive function were reported in two further patients with FIRES on the KDT. SE resolved in both (Singh et al. 2014). In another study, seven patients with FIRES were put on KDT (five via the enteral and two via the intravenous route) with good results. Early initiation of KDT was associated with a favorable prognosis (Peng et al. 2019).

In immune encephalitis associated with drug-resistant epilepsy as well as Rasmussen syndrome, KDTs may also be an option (Caraballo et al. 2013; Appavu et al. 2016). In a study of 10 adult patients managed with the MAD in the chronic management of post-encephalitic epilepsy and autoimmune-associated epilepsy (AE) treated with the modified Atkins diet (MAD), three patients (30%) became seizure-free, one patient (10%) achieved 90% seizure freedom, three patients (30%) achieved a 50–75% reduction in their baseline seizure frequency, and three patients (30%) had no significant benefit. Four patients had either confirmed or presumed viral encephalitis, five patients had seronegative AE, and one patient had GAD65 AE (Husari and Cervenka 2021).

There are different reports of KDT being of benefit in drug-resistant epilepsy due to focal lesions, including hypothalamic hamartoma (Chapman et al. 2011), lissencephaly, bilateral perisylvian polymicrogyria, hemispheric dysplasia, hypoxic ischemic encephalopathy (Thammongkol et al. 2012), and focal cortical dysplasia (Jung et al. 2008). In a recent study, the effectiveness of the diet resulted in decreased seizure frequency and better quality of life in patients with drug-resistant epilepsy secondary to malformations of cortical development, although the authors also stated that seizure freedom is rarely achieved. The diet worked best in patients with unilateral or bilateral polymicrogyria, focal cortical dysplasia not amenable to surgery, and schizencephaly (Pasca et al. 2018).

Of course, children with a clear epileptogenic focus benefit more from resective surgery than from KDTs. Nevertheless, in these cases, KDTs may be used in combination with ASMs to reduce seizures as a bridge to surgery or in patients that are resistant to surgical treatment.

22.7 The Use of the Diet in Status Epilepticus

Over recent years, the diet has been increasingly used in refractory status epilepticus and super-refractory status epilepticus (RSE/SRSE) (Cervenka et al. 2011; Thakur et al. 2014; Wusthoff et al. 2010) both in children and in adults.

In a systematic review of the use of KDT for SRSE, 147 children with SRSE were started on KDT. The diet was initiated at a mean of 5.3 days (range 1–420) after SE onset. In 85/141 (60%) of children, SRSE resolved after a mean of 6.3 days (range 0–19) after SE onset. Response to KDT was more likely when initiated earlier ($p = 0.03$) and in females ($p = 0.01$) (Schoeler et al. 2021).

Analyzing the electroclinical experience in the use of KDTs for RSE, it was observed that children with refractory focal and less frequently generalized SE, primarily observed in focal epilepsies of different etiologies, respond particularly well to the diet. Other types of RSE with a good response to the diet are the pure form of myoclonic SE and non-convulsive and/or electrical SE in the context of epileptic encephalopathies (Caraballo 2018, 2019).

The majority of the studies were conducted in patients with motor RSE associated with an immune-mediated etiology. Patients with focal non-convulsive RSE and generalized RSE have also been shown to respond well to the diet (Thakur et al. 2014; Wusthoff et al. 2010; Cervenka et al. 2011; Kumada et al. 2010). A study on the use of the KD in myoclonic RSE showed a good response to the diet leading to a decrease in ventilation time and the number of ASMs (Caraballo et al. 2017). Two studies evaluating patients with non-convulsive and/or electrical SE treated with KDT found good results (Reyes et al. 2015; Pasca et al. 2018).

Although in children with RSE and SRSE, the effectiveness of KDTs may be difficult to evaluate due to the simultaneous use of other therapies (Peng et al. 2019), different pediatric series found a success rate of around 75%. In responders, the diet worked within 7–10 days (Cervenka et al. 2011; Appavu et al. 2016; Arya et al. 2018).

The use of the diet for the emergency treatment of RSE and SRSE allows for control of the seizures and improvement of the general condition of the patient and may therefore also be used as underlying treatment or for short periods as a bridge to other therapies (Caraballo et al. 2017) (Table 22.4).

Table 22.4 Use of ketogenic diet therapies in different types of status epilepticus

Type of status epilepticus
Focal status epilepticus
Generalized status epilepticus
Myoclonic status epilepticus
Non-convulsive and electrical status epilepticus (epileptic encephalopathies)

Caraballo (2018)

22.8 Management of Ketogenic Diet Therapies

It is recommended to initiate KDT after two or more ASMs have failed (Kossoff et al. 2018); however, the diet may be tried earlier in the treatment scheme, in different syndromes that have been shown to respond well. In addition, KDTs may be tried earlier in the course of RSE and SRSE secondary to certain etiologies and emergency settings (Caraballo 2019).

The classic KD may be started in the hospital or as an outpatient. The advantages of in-hospital initiation are the possibility of close monitoring of the child and teaching on the management of the diet and monitoring of ketosis to the caregivers (Kossoff et al. 2018). However, outpatient initiation may be less stressful. Recent studies show that diet initiation by telemedicine is also feasible in certain situations (Armeno et al. 2022).

Over the years, the diet has become less strict. Where the early protocols were restrictive with prolonged fasting, today the diet is often started on an outpatient basis without the need for fasting, (Bergqvist et al. 2005; van der Louw et al. 2019) and while initially, all foods had to be carefully weighed, currently there are KDTs with “free foods,” such as the MAD (Park et al. 2018) or formulas (Kossoff et al. 2004) and parenteral ketogenic solutions (Armeno et al. 2019). Nevertheless, in certain cases, such as in RSE, fasting may still be useful as it leads to a quicker onset of seizure reduction (Kossoff et al. 2018).

KDTs are used in children whose seizures have failed to respond to ASMs and are usually used in combination with these drugs (Kossoff et al. 2018). If the patient responds well to the diet, the ASMs the patient is receiving may be reduced or withdrawn. However, no clinical guidelines are available and dose reduction is managed according to the clinical criteria used by each epilepsy center (Coppola et al. 2010). A recent study found that reduction of ASMs may be achieved in two-thirds of patients and even complete discontinuation of all ASMs is sometimes possible (Gogou et al. 2022).

Currently, there is no evidence of an interaction between the KD and ASMs, either positive or negative. Therefore, and based on almost 40 years of working with the diet, we consider it important to evaluate each patient individually considering seizure types, type of epilepsy or epileptic syndrome, and etiology. In addition, the treating neurologist should be sure the patient was correctly diagnosed and medicated before prescribing the KD.

Children should be followed by the ketogenic diet team, consisting of a child neurologist, pediatrician specialized in nutrition, nurse, and dietitian, and side effects should be monitored and lab tests requested at each visit. Children should be seen at 1, 3, 6, 9, and 12 months in the first year and every 6 months after that (Kossoff et al. 2018). For adolescent patients on KDT, an adult epilepsy diet center should be available when transitioning into adulthood.

During KDT, the nutritional status of the children should be assessed, making continuous adjustments in caloric intake and supplementation with micronutrients and minerals due to the unbalanced nature of the diet. The ketogenic diet is typically

discontinued after 2 or 3 years; however, in some children, the KD can be extended for more than 10 years.

It is important to always keep in mind that the accurate indication of the diet is the key to success.

22.9 Adverse Effects

Adverse effects may be divided into short- and long-term effects. The most common short-term adverse effects of KDT are gastrointestinal and include dehydration, nausea, vomiting, and constipation. These symptoms are usually mild and easy to correct with adequate fluid and electrolyte intake. Headache, fatigue, dizziness, and insomnia may also occur (Caraballo 2017; Caraballo and Armeno 2023).

Long-term adverse effects include hepatic steatosis, hypoproteinemia, and vitamin and mineral deficiencies. Kidney stones are observed in 3–10% of the patients. Increased serum triglycerides and total and low-density lipoprotein cholesterol levels have been reported in 14–59% of children; however, the serum lipid levels often normalize and remain within normal limits.

On the other hand, there is still a gap in the knowledge regarding long-term complications, such as growth and cardiovascular alterations (Armeno et al. 2019).

In general, the risk of serious adverse effects is low. In the majority of the adverse effects, there is no need to discontinue the diet (Caraballo and Armeno 2023).

22.10 Neuroprotective and Epigenetic Effects of KDT

In our first study of the use of KDT in children with DS, we found that even in patients in whom the reduction in seizures was not dramatic, an improvement in quality of life was seen. In all the children the number of ASMs could be reduced to one or two and one of our patients did not show any further mental deterioration (Caraballo et al. 2005). A subsequent study in an animal model of DS using *Scn1a*-mutant mice showed a neuroprotective effect of the diet (Dutton et al. 2011).

Recent studies support epigenetic mechanisms, such as DNA methylation, histone acetylation, and non-coding RNA, to play a role in addition to the neuroprotective mechanisms of KDT. Interestingly, patients with epilepsy who become seizure-free on KDT may remain so even after discontinuation of the diet, which suggests a disease-modifying mechanism (Murugan and Boison 2020).

Different mechanisms of action may explain the genetic and epigenetic effects of the diet. Neurodegeneration and selective loss of certain populations of neurons, in particular GABAergic interneurons, is pathognomonic of epilepsies. The KDT-induced neuroprotection may be one of the mechanisms underlying the antiepileptic properties of the diet (Murugan and Boison 2020). In addition, the main ketone bodies BHB and acetoacetate are found to have potential neuroprotective

properties. BHB and acetoacetate are also known to have direct effects on specific inflammatory proteins, transcription factors, reactive oxygen species (ROS), and the mitochondria and cause epigenetic modifications.

For parents, cognitive improvement is an essential goal of KDT (Farasat et al. 2006). In our clinical practice, we also see that for parents of children with drug-resistant epilepsy patients, the cognitive benefits are one of the main motivating factors for starting and continuing KDT. Improved cognition and behavior may be an important stimulation for the parents to make the effort to keep their children on the diet.

22.11 Conclusion

KDTs remain the most important non-pharmacological treatment for drug-resistant epilepsy. It is now considered that KDTs should be used earlier in the treatment algorithm for several syndromes.

Different types of epilepsies, mainly the epileptic and developmental encephalopathies are generally drug-resistant. KDTs may work best according to etiologies. Further research, especially in the field of genetics, will be necessary for a better selection of candidates for the diet. In many patients, the diet not only improves epilepsy but also cognition and behavior.

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Chapter 23

Modulating P-glycoprotein Regulation as a Therapeutic Strategy for Pharmaco-resistant Epilepsy



Daniel Perez-Perez, Hiram Luna-Munguia, and Heidrun Potschka

Abstract Experimental data suggest a role of blood–brain barrier P-glycoprotein overexpression as a factor contributing to drug-resistant epilepsy. Therefore, efforts are made to develop and validate therapeutic approaches that aim to overcome transporter-mediated drug resistance. Considering the physiological function of efflux transporters, it will be advantageous to preserve the basal transport function. Thus, recent studies focused on the elucidation of key signaling factors driving P-glycoprotein upregulation in response to epileptic seizure activity. Based on these investigations, novel concepts have been developed: blocking the signaling pathway and controlling P-glycoprotein expression despite recurrent seizure activity. Further development of respective approaches is based on ongoing studies that explore the relevance of the signaling mechanisms in human capillaries and preclinical animal models. In general, the success of respective strategies will depend on the question of whether patients exist in which P-glycoprotein overexpression constitutes a predominant factor contributing to therapeutic failure.

Keywords P-glycoprotein · Efflux transporters · Glutamate · Cyclooxygenase-2 · Drug-resistant Epilepsy

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23.1 Introduction

P-glycoprotein-mediated efflux transport of different antiseizure medications (ASMs) at the blood–brain barrier is considered a limiting factor in ASM brain penetration (Löscher et al. 2011, 2020). The upregulation of these transporters in the epileptic brain might therefore contribute to therapeutic failure.

The hypothesis has been supported by several rodent studies demonstrating a functionally relevant contribution to drug resistance in seizures or epilepsy (Höcht et al. 2007; Potschka 2010a; Wang et al. 2016).

Clinically, overexpression of P-glycoprotein has been repeatedly reported in surgical specimen from patients with drug-resistant epilepsy (Tishler et al. 1995; Aronica et al. 2003; Sisodiya et al. 2006; Hartz et al. 2017). However, data confirming a functional relevance in patients with refractory epilepsy are still limited. Thus, further studies are necessary to explore the relationship between P-glycoprotein overexpression and pharmacoresistant epilepsy.

Population genetic studies have shown controversial results when comparing certain single nucleotide polymorphisms of the P-glycoprotein encoding gene and the responsiveness to specific ASMs (Zhao et al. 2020, 2022; Maqbool et al. 2021; Zhang et al. 2021). In this context, it needs to be considered that these studies did not directly explore P-glycoprotein function. Moreover, it should be taken into account that seizure activity induced P-glycoprotein expression and that the difference between ASM responders and nonresponders might rather be related to genetic and epigenetic differences in regulatory factors triggering P-glycoprotein overexpression.

In addition, drug resistance must be considered a multifactorial problem to which several other factors contribute, including target and network alterations. Moreover, etiology and intrinsic severity can affect pharmacosensitivity (Löscher et al. 2020).

Considering the multifactorial nature of drug resistance, the success of any strategy targeting P-glycoprotein, as one of the efflux transporters, will depend on the question of whether patients exist with a predominance of P-glycoprotein overexpression among different resistance factors. Moreover, it will be necessary to identify respective patients based on biomarkers such as positron emission tomography (PET) with P-glycoprotein substrate radiotracers.

23.2 Strategies to Overcome P-glycoprotein-Mediated Efflux Transport

During the past two decades, several strategies have been discussed to overcome the P-glycoprotein-mediated efflux transport of ASMs (Potschka 2010a).

One obvious strategy is based on pharmacological inhibition and modulation of P-glycoprotein function. Whereas first- and second-generation inhibitors of P-glycoprotein (e.g., verapamil, cyclosporin A, and valsopodar) were characterized

by additional pharmacodynamic and pharmacokinetic effects, which might hamper their use, more selective and potent third-generation inhibitors (e.g., tariquidar, elacridar, and zosuquidar) have been developed (Thomas and Coley 2003). These efforts in particular aimed to develop compounds for combination with cytostatic drugs in cancer patients in which transporter overexpression contributes to therapeutic failure. However, clinical studies have failed related to a relevant increase of the toxic effects of the cytostatic drugs. This increase in toxicity is likely related to enhanced distribution across various sensitive tissues and cells, which are known to be protected by P-glycoprotein from exposure to harmful xenobiotics (Fox and Bates 2007).

In general, long-term add-on therapy with potent P-glycoprotein modulators in patients with drug-resistant epilepsy needs to take into account that this will limit the protective function of P-glycoprotein throughout the body. Therefore, one should only consider an interval therapy with a transient add-on of P-glycoprotein modulators until P-glycoprotein expression returns to control levels and ASMs are efficacious again when administered alone. However, this hypothetical regime has not been validated experimentally or clinically.

So far, experimental evidence has shown that tariquidar add-on treatment can help to overcome drug resistance in chronic models of drug-resistant temporal lobe epilepsy in rats (Brandt et al. 2006). Clinical experience with P-glycoprotein inhibitors is limited to small clinical trials and case reports. These reports cannot be interpreted clearly as verapamil, which possesses additional pharmacodynamic and pharmacokinetic effects, has been used for P-glycoprotein modulation, and patient selection was not based on P-glycoprotein expression rates (Summers et al. 2004; Iannetti et al. 2005, 2009; Asadi-Pooya et al. 2013; Nicita et al. 2014; Borlot et al. 2014; Narayanan et al. 2016; Elkhayat et al. 2017; Lakshmikanthcharan et al. 2018). Concerning the use of verapamil as a P-glycoprotein competitive inhibitor, a note of caution is necessary considering that testing an add-on therapy in a pilot translational study in canine patients with drug-resistant epilepsy indicated that verapamil might also aggravate seizure control (Jambroszyk et al. 2011). In this context, it might be relevant that substrates of P-glycoprotein must not necessarily competitively inhibit each other's transport (Sikri et al. 2004). Depending on the interaction site in the binding pocket of P-glycoprotein, two substrates might also be co-transported efficaciously (Sikri et al. 2004; Subramanian et al. 2016; Mollazadeh et al. 2018).

As an alternate approach, bypassing efflux transporters or bypassing the blood-brain barrier might be considered (Potschka 2010b). Nanoparticle encapsulation of ASMs has been suggested as one approach. However, brain penetration and distribution rates are still not satisfying, so further efforts seem to be necessary to optimize respective delivery tools. Local administration into the epileptogenic zone is characterized by the invasiveness of the approach.

As another strategy, noninvasive neuromodulation is a technique in which the patients are exposed to a low-intensity electrical field that modifies their brain activity (Liu et al. 2018). Preclinical experience provided evidence that this add-on therapeutic technique can also modulate P-glycoprotein expression and function

(Pérez-Pérez et al. 2021). Nevertheless, the clinical evidence for this technique is still very limited (Boon et al. 2018).

Considering the disadvantages or limitations of the different strategies, efforts were made to develop further alternatives. In particular, it was tried to identify targets in signaling pathways contributing to P-glycoprotein upregulation in the epileptic brain. These targets might help to develop add-on strategies preventing overexpression of P-glycoprotein in epilepsy patients (Potschka 2010c).

23.3 Regulation of P-glycoprotein Expression

To develop approaches that prevent upregulation of P-glycoprotein, it was crucial to identify key signaling factors that contribute to P-glycoprotein regulation in response to epileptic seizure activity (Fig. 23.1). Glutamate was among the main candidate factors due to its high ictal extracellular concentrations and first evidence that glutamate might affect P-glycoprotein expression in brain capillaries. Studies using *ex vivo* preparations of rodent brain capillaries exposed to high glutamate concentrations confirmed that this neurotransmitter can trigger transcriptional activation of the P-glycoprotein encoding gene resulting in enhanced functional surface expression of the efflux transporter (Bauer et al. 2008). The *in vivo* role was substantiated by injecting glutamate in the right hippocampus of rats in concentrations that did not induce electrographic or behavioral seizure activity (Bauer et al. 2008). In this study, glutamate induced a local upregulation of brain capillary P-glycoprotein expression. The effect of the neurotransmitter proved to be mediated by NMDA receptor signaling as an antagonist of this receptor prevented the impact of glutamate on P-glycoprotein (Bauer et al. 2008).

NMDA receptor activation seems to mediate its effects on transcriptional activation of the P-glycoprotein encoding gene via an intracellular cascade that involves the release of arachidonic acid from the cell membrane (Potschka 2010c). In this context, the role of the inflammatory enzyme cyclooxygenase-2 has been confirmed based on pharmacological inhibition as well as genetic deficiency studies in isolated

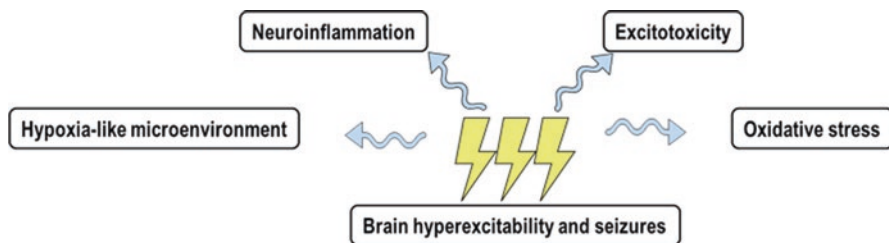


Fig. 23.1 Biological processes in response to epileptic seizure activity contributing to P-glycoprotein overexpression. (Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License)

rodent brain capillaries (Bauer et al. 2008; van Vliet et al. 2010). In contrast, pharmacological modulation of cyclooxygenase-1 had no impact on glutamate-mediated increases of P-glycoprotein.

Among the prostanoid products of arachidonic acid signaling, prostaglandin E2 (PGE₂) and its receptor (EP1) were identified as further key elements in the P-glycoprotein regulatory signaling pathway (Pekcec et al. 2009).

Recurrent seizure activity in the epileptic brain has also been related to the induction of a hypoxia-like cellular environment. This environment is associated with a higher expression of hypoxia-inducible factor 1 alpha (HIF-1 α), a key signaling promoter of P-glycoprotein expression (Merelli et al. 2019; Wang et al. 2019).

In addition, activation of inflammation-related pathways via the Toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF- κ B) can also be associated with an increased expression of P-glycoprotein (Wang et al. 2017; Tang et al. 2022). Moreover, these inflammatory markers seem to be useful biomarkers for the severity of the disease (Kamaşak et al. 2020; Vega-García et al. 2021).

23.4 Targeting Signaling Pathways of P-glycoprotein

The elucidation of the key signaling factors, which drive P-glycoprotein expression in the epileptic brain raised the main question whether targeting of these factors might help to control P-glycoprotein expression at control levels and might help to overcome drug resistance (Fig. 23.2).

In this sense, the cyclooxygenase-2 inhibitor celecoxib proved to be efficacious in a rat electrical *status epilepticus* model by preventing the seizure-associated P-glycoprotein up-regulation in different hippocampal and cortical subregions (Zibell et al. 2009). Interestingly, a later study conducted in a rat chronic epilepsy model with spontaneous recurrent seizures, confirmed that cyclooxygenase-2 inhibition can also help to bring back brain P-glycoprotein expression rates to control levels (van Vliet et al. 2010). In the same experimental setup, pharmacological targeting of cyclooxygenase-2 increased the brain penetration rate of the antiepileptic drug phenytoin (van Vliet et al. 2010).

As the next experimental step, phenobarbital nonresponders were selected in a chronic model with recurrent spontaneous seizures. Following the selection procedure, rats were treated for 6 days with the cyclooxygenase-2 inhibitor celecoxib. Then, following the withdrawal of celecoxib, the efficacy of phenobarbital was tested again in the same group of animals (Schlichtiger et al. 2010). In this second drug treatment phase, seizure control was significantly improved as a consequence of celecoxib pretreatment. In addition to the impact on P-glycoprotein further effects of the anti-inflammatory treatment might have contributed to the therapeutic success in this experimental setup. In particular, it is known that inflammatory processes can contribute to ictogenesis, might enhance signaling via glutamate receptors, and decrease GABAergic signaling due to an impact on

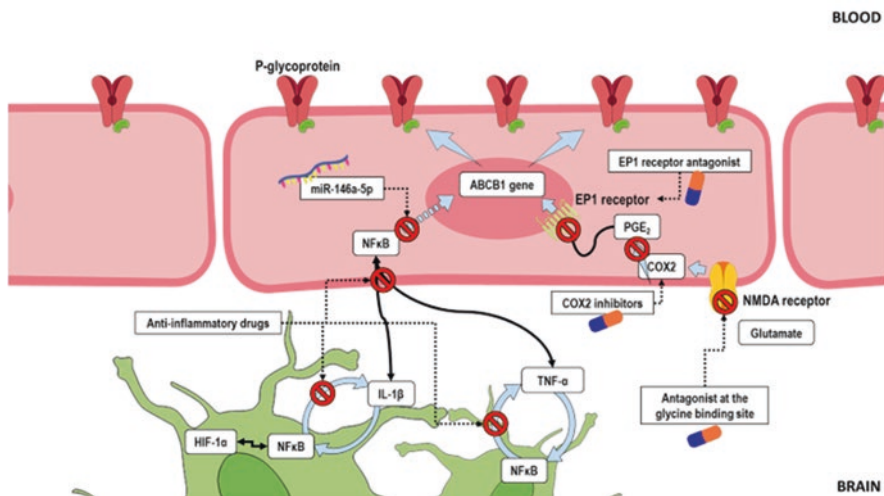


Fig. 23.2 Signaling pathways that upregulate P-glycoprotein in response to seizure activity. Preclinical evidence on targeting these pathways is displayed. IL-1 β interleukin 1 beta, TNF- α tumoral necrosis factor alpha, ABCB1 the coding gene of P-glycoprotein, COX2 cyclooxygenase 2. Dashed arrows connect the therapy with its target; continuous and blue arrows indicate pathways; stop symbols indicate a blockage. (Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License)

receptor subunit expression or due to a modulation of the functional state of the receptors (Vezzani et al. 2013).

Although these studies show promising preclinical results, a recent veterinary clinical trial using an add-on therapy with firocoxib (a cyclooxygenase-2 inhibitor), demonstrated a low success rate in canine patients with drug-resistant epilepsy after six months of treatment (Fischer et al. 2022). Interestingly, the only two dogs that benefited from the therapy were those with the highest seizure frequency at the beginning of the study (Fischer et al. 2022). These findings suggest that only a subgroup of patients may benefit again indicating that a selection procedure is required to stratify patients that might benefit from strategies preventing P-glycoprotein induction.

Targeting of the EP1 receptor was further confirmed as an alternate approach to cyclooxygenase-2 inhibition. EP1 receptor antagonism kept P-glycoprotein expression at control levels in a rat *status epilepticus* model (Pekcec et al. 2009). Moreover, subchronic treatment with an EP1 receptor antagonist during a massive kindling phase with frequent elicitation of seizures improved the efficacy of phenobarbital on kindled seizures evaluated following withdrawal of the EP1 receptor antagonist. However, the efficacy in a model of drug-resistant epilepsy still needs to be evaluated.

More recently, inhibition of the microsomal PGE2 synthase-1 has been described as an alternative strategy, which was assessed in isolated brain capillaries and in a *status epilepticus* model in humanized mice (Soldner et al. 2019).

In addition to targeting the arachidonic acid signaling cascade, we might also consider targeting the NMDA receptor (Bankstahl et al. 2008; Bauer et al. 2008). However, earlier experimental and clinical studies have demonstrated that epilepsy causes a significantly enhanced sensitivity to the side effects of competitive and noncompetitive NMDA receptor antagonists (Löscher and Hönack 1991a, b; Sveinbjornsdottir et al. 1993). Thus, translational development of respective experimental approaches, which proved that blocking of the NMDA receptor-associated ion channel controls P-glycoprotein expression during a *status epilepticus*, cannot be considered based on tolerability issues. Targeting the co-agonist glycine-binding site of the NMDA receptor might render an alternate target, which needs to be further studied (Avemary et al. 2013; Zellinger et al. 2014).

Preclinical studies demonstrated that silencing mRNA targeting the inhibitory κ B kinase subunit β (relevant regulator on the NF- κ B pathway) can prevent P-glycoprotein overexpression in a chronic rat epilepsy model (Yu et al. 2014). More recently, upregulation of the regulatory microRNA miR-146a-5p has been linked with a reduction in the expression of different components of the NF- κ B and interleukin-1 β signaling pathway and of P-glycoprotein (Deng et al. 2019). Their findings also suggested a potential therapeutic effect of a miR-146a-5p mimic (Deng et al. 2019). Further support for the beneficial effects of this miRNA mimic came from a preclinical study in a mouse model with carbamazepine-resistant seizures (Iori et al. 2017). However, the authors only assessed the effects of the miRNA mimic without additional analysis of whether this molecule would also restore the efficacy of carbamazepine.

Interestingly, efforts to develop dual-action antiseizure and anti-inflammatory compounds have shown beneficial effects in animal models, but clinical validation of respective compounds has not been reported yet (Enrique et al. 2021). In this context, it is of interest that two clinical trials evaluating the effects of methylprednisolone (NCT04219995) and aspirin (NCT03356769) in epilepsy were registered on clinicaltrials.org, but no information on the outcome is yet available. A study in veterinary medicine demonstrated that the use of a cannabidiolic acid (CBDA) and cannabidiol-enriched oil significantly reduces seizure frequency in dogs with drug-resistant seizures (Garcia et al. 2022). A possible explanation for these effects may lie in dual actions of the compounds, with a COX-2 inhibitory effect of cannabidiolic acid blocking P glycoprotein signaling on one hand and cannabidiol blocking the P-glycoprotein function on the other hand (Takeda et al. 2008; Auzmendi et al. 2020). However, considering the rather weak effect of CBDA on COX-2, it seems necessary to further evaluate its functional significance.

In addition, it is necessary to rule out species differences in the signaling pathways. Therefore, studies were completed to assess the regulation in brain capillaries prepared from surgical specimens dissected from patients with drug-resistant epilepsy. These data confirmed that the same signaling pathway involving an endothelial NMDA receptor and cyclooxygenase-2 drives P-glycoprotein overexpression in

human capillaries (Avenary et al. 2013). While subsequent studies demonstrated that the glutamate-induced NMDA receptor/cyclooxygenase-2 signaling pathway also induces expression of multidrug-resistance associated family member 2, an opposite effect was observed for breast-cancer resistance-associated protein 2 (ABCG2) (Luna-Munguia et al. 2015; Salvamoser et al. 2015).

Based on a series of more recent studies that explored the mechanisms regulating the trafficking of P-glycoprotein, it has been discussed that targeting intracellular and intercellular trafficking mechanisms may also serve as potential targets for the management of P-glycoprotein-related drug resistance (Löscher and Gericke 2020).

Considering that the clinical data supporting a functional relevance of transporter overexpression is still limited, further assessment of the impact on patients will be necessary. Moreover, taking the multifactorial nature of drug resistance into account it needs to be determined whether a subgroup of patients exists in which a specific resistance mechanism predominates.

23.5 Biomarkers of P-glycoprotein-Associated Drug Resistance

As outlined above further assessment of the relevance of P-glycoprotein upregulation in patients is of utmost importance. In addition, a tool is needed to identify patients with P-glycoprotein overexpression for any application of therapeutic approaches aiming to overcome transporter-mediated resistance.

Considering the complexity of the regulatory events driving P-glycoprotein expression in the epileptic brain, it is unlikely that genetic analyses will be helpful in this context (Potschka 2010d). PET has been suggested as an imaging tool for the analyses of blood–brain barrier P-glycoprotein function based on a clinical pilot study using [11C] verapamil (Langer et al. 2007). Meanwhile, the concept has been developed further with the performance of two subsequent scans using a P-glycoprotein substrate radiotracer with or without administration of a pharmacological P-glycoprotein modulator. In one of these studies, [18F] MPPF has been validated as a suitable radiotracer. In control rats, tariquidar pretreatment significantly affected the influx and efflux rates of [18F] MPPF confirming that the tracer is subject to blood–brain barrier efflux transport mediated by P-glycoprotein (la Fougère et al. 2010). Based on these findings, the tracer kinetics were compared between phenobarbital responder and nonresponder rats in a chronic epilepsy model. The findings revealed that in line with the hypothesis, nonresponder rats exhibited a more pronounced response to the P-glycoprotein modulator tariquidar with influx and efflux rates of [18F] MPPF more intensely affected in nonresponders as compared to responders (Bartmann et al. 2010).

Based on these data respective PET studies have been performed in patients. The P-glycoprotein function has been evaluated in patients with temporal lobe epilepsy and with focal cortical dysplasia using (R)-[11C]-Verapamil as a radiotracer. The

authors inferred higher P-glycoprotein function in the epileptogenic brain areas of the patients from lower uptake of the radiotracer compared with controls (Feldmann and Koepp 2012; Ilyas-Feldmann et al. 2020). Moreover, they reported a different extent of the influence of the P-glycoprotein inhibitor tariquidar on (R)-[11C]-Verapamil uptake.

While interpreting the patients' PET findings, we have to consider several factors such as the differences observed in the pathology itself and the treatment regime. Together, this can induce certain variability in the tracer's brain penetration and its modulation by tariquidar. In particular, it remains difficult to predict the interaction between the tracer, the modulator, and the ASMs. As mentioned above, the large binding pocket of the transporter molecule allows different scenarios in the interaction between compounds that bind to P-glycoprotein in parallel. Some might competitively inhibit each other's binding and transport, whereas others might be co-transported.

23.6 Future Perspectives

Whereas experimental data confirm a contribution of the efflux transporter P-glycoprotein to drug resistance, respective clinical data are still rather limited. Thus, further studies are urgently needed to finally conclude about the functional relevance in the clinical setting. Moreover, translational development of any strategy overcoming efflux transport is based on the assumption that a subgroup of patients exists in which this mechanism of resistance predominates among other mechanisms such as network and target alterations. Provided that clinical proof-of-principle is obtained in the future, selecting patients with transporter overexpression for respective clinical studies will be necessary.

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Chapter 24

Vagus Nerve Stimulation for Intractable Seizures



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Abstract Vagus nerve stimulation (VNS) is the most widely used non-pharmacological treatment for drug-resistant epilepsy (DRE) since its approval by the FDA (1997) “as an adjunctive treatment for partial refractory epilepsy in adults and adolescents over 12 years of age.” Given the better definition and understanding of DRE and the need for options -even if only palliative- to address its devastating health, psychosocial and economic consequences, VNS usage was extended to younger age groups and patients with generalized seizures. VNS therapy involves the implantation of a battery-operated device in the upper chest with two subcutaneously placed wires with electrodes attached to the left vagus nerve in the carotid sheath. Stimulation is usually initiated 15 days after implantation and adjusted over time based on patient tolerance and response. The safety, tolerability, and efficacy of the procedure and therapy have been repeatedly demonstrated in prospective randomized clinical trials, uncontrolled retrospective series, and long-term follow-up series. Complications of surgery are rare with infection being the most often reported, while stimulation-related side effects are usually mild and, mostly, decrease over time. Series have shown a remarkably consistent average reduction in seizure frequency of 40–50% responder rate (i.e., the proportion of patients whose seizure frequency is reduced by at least 50%) with no obvious indication of tolerance and generally a long-term increase in efficacy. The number of patients who become seizure-free is relatively small. Other positive outcome measures include improvement in mood, alertness, memory, and postictal recovery period. The most recent addition to this therapy has been cardiac-based seizure detection, which involves monitoring heart rate to elicit an additional train of stimulation. Cost-benefit has been documented, although in many countries the deterrent to the use of VNS is the initial cost of the device. Despite extensive animal and clinical studies, as well as innovations in software and hardware the exact mechanisms of action, definition of optimal stimulation parameters and duty cycles, and precision of outcome predictive factors requires further exploration. VNS should be considered

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within a comprehensive epilepsy surgery center on a patient-to-patient basis, following a detailed bio-psycho-social workup and review of expenses and risks weighted against expectations, and presumed outcomes.

Keywords Vagus nerve stimulation · Intractable seizures · VNS mechanisms of action · Stimulation parameters · Seizure outcome · Other measures of outcome · Predictive factors

24.1 Introduction

During the 25 years since the FDA approved vagus nerve stimulation (VNS) therapy in 1997, and its earlier approval in the European community in 1994, VNS has become a mainstay in the comprehensive treatment of drug-resistant epilepsy (DRE). In a book dealing with pharmacoresistance in epilepsy, the reason for this trend should be fairly evident, since we are faced with the reality (also evident and permanently latent) that there are despite new antiseizure medications (ASM) and dramatic improvements in resective surgical procedures still many patients with refractory epilepsy who do not benefit from any of these advances and carry a great burden on quality of life and overall cost of the disease. As other neuromodulation techniques are explored for these subsets of DRE patients, VNS, which consists of the chronic and intermittent stimulation of the vagus nerve (VN) in its intracranial cervical segment and was the first of FDA approved device has become mainstream in DRE treatment. VNS therapy has been used to treat more than 1,25,000 people with DRE, including 35 children (Livanova data <https://www.livanova.com/epilepsy-vnstherapy/en-us>). Over the past two decades, the technology has evolved through multiple iterations resulting in software-related updates and implantable lead and generator hardware improvements. The safety and effectiveness of the procedure were established in prospective randomized clinical trials and uncontrolled retrospective series (Ben Menachem 2001; Schachter and Wheless 2002), showing a remarkably consistent average reduction in seizure frequency of 40–50% responder rate (i.e., the proportion of patients whose seizure frequency is reduced by at least 50%) with no obvious indication of tolerance. The most recent addition to this therapy has been cardiac-based seizure detection (CBSD), which involves monitoring heart rate to elicit an additional train of stimulation when rapid heart-rate accelerations (often associated with seizures) are detected (Tzadok et al. 2019). However, there are basic considerations that render the precise role of VNS in the treatment of DRE, as yet, uncharacterized and, practically, a palliative approach, (a) the fact that the pathophysiology of stimulation remains elusive and is surely based on more than one mechanisms and dependent on parameters of stimulation and duty cycles and the dynamic state of the target neurons, and (b) given this uncertainty the precise definition of stimulation protocols/parameters and identification of factors that reliably predict its effectiveness remains elusive. Hence, the most important

consideration to keep in mind is that VNS should be considered within a comprehensive epilepsy program as an option based on *exclusion criteria*: the selection should be assessed on a patient-to-patient basis, ensuring that potential benefits on seizure frequency and/or severity and quality of life, justify the risks and expense of VNS therapy.

24.2 Mechanisms of Action (MOA)

The mechanisms of VNS leading to seizure suppression remain undefined, despite numerous experimental and clinical studies focusing primarily on neurophysiology, neuroanatomy, neurochemistry, cerebral blood flow (CBF), and functional brain connectivity studies. Expanding on the work of Bailey and Bremner in the 1930s and Dell, Olsen, and Zanchetti in the 1950s, Zabara proposed that applying intermittent electrical current to the cervical VN would “desynchronize” cerebral cortical activity, thereby attenuating seizure frequency (Hammond et al. 1992). It was therefore assumed VNS would produce changes in the electroencephalogram (EEG) in humans, and many initial studies focused on these changes (Kuba et al. 2002). However, no conclusive changes in basal activity have been demonstrated. Investigators have reported EEG changes during sleep and awake states, as well as acute and chronic changes in EEG and evoked potentials (Marrosu et al. 2005; Hammond et al. 1992). Koo (2001) documented a progressive EEG change characterized by the grouping of epileptic activity followed by increasingly longer periods without seizures. One study that evaluated interictal epileptiform discharges documented an important decrease in such discharges when compared to a basal recording without VNS. The differences in animal and human effects of VNS may be largely explained because VNS efficacy in animals has been primarily assessed in acute models (3-mercaptopropionate, pentylenetetrazole, maximal electroshock, penicillin, or strychnine application), and only a few studies have used animal models of chronic epilepsy (Lockard et al. 1990; Muñana et al. 2002). In the rodent model, mimicking epilepsy in humans, the Genetic Absence Epilepsy Rats of Strasbourg, acute VNS applied shortly after the onset of Spike wave discharges (SWD) prolonged the mean duration of SWD during the first day of VNS, but chronic stimulation hardly affected SWD (Dedeurwaerdere et al. 2005). It has also been shown that VNS exerts a powerful acute anticonvulsant effect on spontaneous seizures occurring in rats, previously submitted to full electrical kindling of the amygdala. This VNS-treated kindled rat model has been proposed as clinically relevant since it affects limbic seizures, which are most responsive to VNS in epilepsy patients (Rijkers et al. 2010). Another point that has been made to explain differences in animal and human studies is that in animal experiments the effect of VNS is evaluated when stimulation is performed in close relation to time of seizure onset, testing the anti-seizure effect of VNS. In human beings, VNS is used in an intermittent mode. Seizures are also suppressed when the stimulator is in the “off” mode,

suggesting an anti-epileptic rather than an anti-seizure effect only. This finding is supported by the animal studies by Takaya et al. (Takaya et al. 1996) who demonstrated that the efficacy of VNS outlasts the duration of the stimulation train. There may be different pathways involved in anti-seizure and anti-epileptic effects.

The fact that seizures recur after the end of battery life is reached is a strong argument against VNS having an antiepileptic effect. However, as the development of more relevant animal models progresses, the antiepileptic potential of neuromodulation in general is being explored and some promising results have been reported. VNS significantly delayed amygdaloid kindling (AK) in cats and stage VI was not reached despite 50 AK trials (Fernandez-Guardiola et al. 1999) but this effect was absent when VNS was started after the cats had already reached a more severe seizure stage (Magdaleno-Madrigal et al. 2004). Kindling in rats was slowed as well: 1 h of VNS prior to the kindling pulse increased the mean number of stimuli needed to reach the generalized seizure state (Naritoku and Mikels 1997). These anti-epileptogenic effects of VNS, however, could not be confirmed by another study, where the kindling rate did not differ between animals treated with 2 h of VNS prior to the kindling stimulus and controls (Dedeurwaerdere et al. 2006). The authors suggested that VNS treatment could have rendered the amygdala more excitable because after discharge threshold determination evoked generalized seizures in all VNS-treated animals and only in half of the controls. There is one report of long-lasting seizure control after the explantation of the VNS device (Labar and Ponticello 2003).

Other clinical investigations on EEG synchronization and power spectral analysis have shown that acute VNS stimulation results in desynchronization in theta bands in ECoGs recorded in patients with dual (VNS and responsive neurostimulation) neurostimulators (Ernst et al. 2021) and power spectral analysis revealed significant EEG reactivity to photic and hyperpnea stimuli between responders and non-responders (Brázdil et al. 2019).

The early hypothesis of cortical desynchronization induced by activation of unmyelinated afferent vagal fibers through the reticular activating system was contradicted in human studies, because effective therapeutic parameters were sub-threshold for fibers C. Krahel et al. found strong evidence that vagal fibers C are neither responsible nor necessary for the seizure-suppressing effect of VNS (Krahel et al. 2001). According to their experiments in awake and freely moving cats, activation of myelinated A and B fibers is responsible for seizure suppression. Recent evidence derived from experiments conducted in rodents (Chang et al. 2020) showed a correlation between the type of physiological response and the type of fibers involved. C-fiber activation was strongly associated with breathing changes and apnea. The amplitude required to recruit C-fibers would however also give rise to significant adverse effects. Their rodent model suggests that quantitative estimation of nerve fiber engagement could prove pertinent to refine VNS therapy by tailoring the desired type of fiber involvement to the indication. However, it must be remembered that the different types of fibers making up the trunk of the nerve may be differently organized across individuals and affected differently by the stimulation

depending on pulse width, output current, and electrode design, as modeling simulation suggests.

Other studies have focused on the activation/deactivation of certain brain areas using regional blood flow mapping, single-photon emission computed tomography (SPECT), positron emission tomography (PET), and functional MRI (fMR). Increased bilateral brain activity in the rostral medulla, thalamus, hypothalamus, insula, and postcentral gyrus, with greater contralateral activation has been documented. Reduced perfusion during stimulation in the ipsilateral brain stem and the limbic system has also been evidenced (Barnes et al. 2003). One study carried out with SPECT and EEG during VNS activation/deactivation demonstrated that with short-cycle stimulation (7-s stimulation, 12 s turned off) there was a relative reduction of activation in the medial bilateral thalamus (Ring et al. 2000). Studies carried out using fMR found induced activation by left VNS in the thalamus (bilateral and toward the left side), bilateral insular cortex, postcentral gyrus and ipsilateral basal ganglia, right temporal posterosuperior gyrus and inferomedial occipital gyrus (higher on the left side). The highest activity was found in the left thalamus and insular cortex. This suggests that these areas play an important role in the modulation of brain cortex activity (Narayanan et al. 2002). It is likely that VNS also causes anti-seizure effects at non-thalamic sites, including the locus coeruleus, which produces most of the cerebral norepinephrine (NE), and the raphe nuclei, which produces most brain serotonin. Another structure identified in the anticonvulsant effect of VNS is the nucleus of the solitary tract (NST), which receives 95% of the vagal afferent fibers and is regulated by cholinergic innervation. Moreover, the NST projects the sensory information to different areas of the brain, including the amygdala, cerebellum, hypothalamus, thalamus, parabrachial nucleus, raphe nuclei, and locus coeruleus. Thus, the VN is projecting sensory information via NST to NE and serotonin (5-HT) systems, which are associated with the regulation of mood, anxiety, emotion, and seizure activity. Interestingly, it has been found that changes in γ -aminobutyric acid (GABA)-ergic and glutamatergic transmission in the NST can regulate the susceptibility to seizures (Walker et al. 1999). In particular, an increase in GABA transmission or a decrease in glutamate transmission in the rat NST reduces susceptibility to limbic motor seizures evoked by systemic and focal bicuculline and systemic pentylenetetrazol. However, hippocampal GABA levels were not changed after VNS (Meurs et al. 2008).

A resting-state fMRI functional connectivity study on the brainstem-cortical/subcortical structures in eight controls and eight VNS responders concluded that VNS could reorganize the altered functional connectivity (Fc) between the brainstem and insula, precuneus, and cerebellum (Zhu et al. 2020). Similarly, a study on 66 children with DRE and VNS, compared metabolic connectivity between responders and non-responders using preoperative fluorodeoxyglucose PET. Relative changes in glucose metabolism were strongly connected among the areas of the brainstem, cingulate gyrus, cerebellum, bilateral insula, and putamen in patients in responders. These results support the existence of specific preexisting connectivity patterns in responders vs. non-responders (Yu et al. 2018).

Other investigations on MOA focus on metabolites expressed in VNS cases or activation areas. An increase in 5-hydroxyindoleacetic acid, homovanillic acid, aspartate, 5-HT, and dopamine metabolites, which are significantly associated with seizure control, has been demonstrated. Also, some studies have documented an increase in GABA and ethanolamine in responders. VNS protects cortical glutamic acid decarboxylase (GAD) positive neurons from death subsequent to brain lesions and may increase GAD cell count in the hippocampal hilus of the injured brain (Neese et al. 2007). Using a SPECT study in DRE patients, it was described that VNS may modulate cortical excitability of brain areas associated with epileptogenesis and that GABA-A receptor plasticity contributes to this effect (Marrosu et al. 2003).

Concerning growth factors known to play a crucial role in neuronal tropism, acute VNS in normal rats increases the expression of brain-derived neurotrophic factor (BDNF) and fibroblast growth factor in the hippocampus and cerebral cortex and decreases the abundance of nerve growth factor mRNA in the hippocampus (Follesa et al. 1994). Furthermore, progenitor cell proliferation in the dentate gyrus increases after 3–48 h of VNS (Revesz et al. 2008) and is still detectable 3 weeks later. However, chronic VNS for 4 weeks did not affect the number of proliferating cells (Biggio et al. 2009) nor was the survival of progenitor cells enhanced by VNS (Revesz et al. 2008). Since both NE and 5-HT can influence progenitor cell proliferation (Kulkarni et al. 2002), these VNS-induced plastic changes may be the result of reported neurotransmitter changes.

The VN is also implicated in immunomodulation as efferent vagus nerve fibers systemically inhibit pro-inflammatory cytokine release (Pavlov and Tracey 2005). Although it is still unclear to what extent VNS affects this so-called “cholinergic anti-inflammatory pathway,” VNS appears to exert an afferent neuroimmunomodulatory effect since 2 h of continuous VNS-induced expression of the pro-inflammatory cytokine interleukin-1 β in the hippocampus and hypothalamus of rats (Hosoi et al. 2000). Furthermore, VNS activates the hypothalamic-pituitary-adrenal axis. This was demonstrated by VNS-induced increased hippocampal expression of corticotrophin-releasing factor and increased plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone after VNS (Hosoi et al. 2000).

Given the recent emphasis on brain connectivity analysis and neural networks in epileptogenesis models further research has focused on brain connectivity studies. A pilot study (de Vos et al. 2011) explored predictive interictal EEG features for seizure reduction in 19 patients with medically refractory epilepsy submitted to VNS. They found that a quantitative symmetry measure, the pair-wise derived brain symmetry index (pdBSI), was on average higher for delta, theta, alpha, and beta bands for non-responders (9 patients) than for responders (10 patients). The average pdBSI of the theta and alpha bands could significantly discriminate between responders and non-responders. Fraschini showed that VNS-induced global decrease of functional connectivity in the gamma band was significantly higher in responders than in patients who failed to show improvement after VNS (Fraschini et al. 2013). The analysis was based on the phase lag index (PLI), which allows the study of the global functional connectivity among the EEG sensors and reduces the

effect of volume conduction. In two studies, one on 19 patients (Bodin et al. 2015) and the other on 35 patients (Sangare et al. 2020) with chronic VNS, responders had reduced interictal functional connectivity on scalp EEG than non-responders. The correlation between VNS-induced interictal EEG time-series decrease in functional connectivity and decrease in seizure frequency suggests that the therapeutic effect of VNS may be related to changes in interictal functional connectivity. Reducing the hyperconnectivity found in the epileptogenic zone could be one of the MOA of VNS in focal epilepsies.

More recent studies aiming at predicting VNS response based on structural and functional connectomic profiling have offered insights. VNS responders showed greater fractional anisotropy in the left thalamocortical, limbic, and association fibers and greater connectivity in a functional network encompassing the left thalamic, insular, and temporal nodes, pointing toward a similar network to that of early functional imaging studies. A similar study from the same group had first shown that thalamocortical intrinsic connectivity could play a key role in the preoperative estimated response to VNS, supporting further the existence of a specific network coming into play and affected by VNS (Carron et al. 2022).

Given these experimental observations and the variable clinical response in different patient subgroups (according to age/type of seizures/etiology), it seems plausible to propose that the MOA of VNS involves several neural pathways and networks, with some synergistic actions. The synergistic action might apply to some ASM regimens, as well. A clear understanding of the correlation between the parameters of stimulation and functional connectivity and spatial and temporal synchronization still needs to be established. There is probably no such thing as one single mechanism but several, depending on parameters of stimulation and duty cycle, which significantly vary across studies and are certainly dependent on the dynamic state of the brain when the stimulation reaches the target neurons (Carron et al. 2022).

24.3 Patient Selection and Indications

Physicians often differ about when it is appropriate to begin discussing the possibility of VNS therapy. In 30–40% of epilepsy patients who are evaluated for DRE, there is often a controversy about whether polytherapy or add-on is appropriate after two trials of monotherapy have failed, but most agree that patients with localization-related seizures should be evaluated for epilepsy surgery if the third drug trial fails to control the seizures. It is our practice to follow a protocolized presurgical evaluation in these patients that includes at least history and physical examination, EEG, MR imaging, and in most cases, video-EEG, and functional imaging studies to discuss surgical options only once a detailed description or working hypothesis of the seizure type, epileptic syndrome and possible epileptogenic area has been determined. As stated, the author considers VNS as a resort option when other surgical treatments have been discarded (Fig. 24.1).

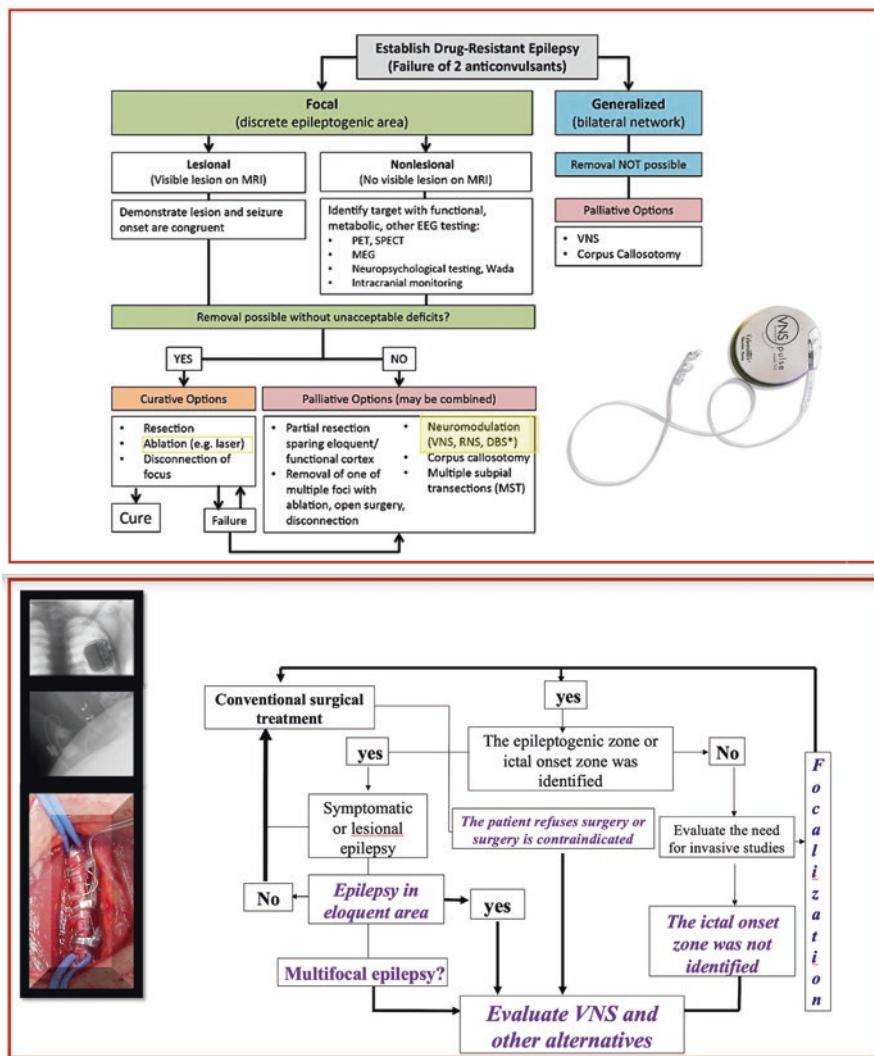


Fig. 24.1 Study algorithm and decision-making of treatment stages and goals

Initially, the FDA approved the use of VNS as an adjunctive therapy in adults and adolescents with partial onset seizures. However, this therapy has been increasingly applied to diverse groups, obtaining benefits in cases with tuberous sclerosis (Parain et al. 2001), Lennox-Gastaut syndrome (Frost et al. 2001), hypothalamic hamartomas (Murphy et al. 2000), ring chromosome 20 syndrome with intractable epilepsy (Chawla et al. 2002), and bitemporal epilepsy (Kuba et al. 2003). Given the more precise definition and relevance of DRE including increased concern about adverse effects of medications on neurological development, VNS usage was extended to patients <12 years old. During the initial experience, it was observed that 61 % of the

pediatric population presented a seizure frequency reduction >50% at 12 months (Wheless and Maggio 2002) and even more favorable rates were suggested as characteristic responses for this population (Murphy et al. 2003). The findings by Thompson et al. (2012) in 146 patients followed up for a mean of 41 months, 32% with partial epilepsy and 68% with generalized epilepsy, showed the reduction in seizure frequency in 91% of patients, seizure duration in 50%, a postictal period in 49%, and ASM use in 75%, clearly suggest limitations on the FDA-approved indications for VNS therapy, which reflect the design of the original preapproval prospective studies, and do not accurately delineate the patient population able to benefit from this approach.

Other scenarios have been considered as possible indications: (a) cases in which patients, for personal reasons emphatically refuse brain surgery; (b) patients with severe epilepsy in whom surgery carries a significant risk of failure and/or functional postoperative deficits and caregivers decide that the expense of VNS is preferable as an initial option; and (c) selected cases with failed surgical results (Amar et al. 2008; Vale et al. 2011).

Another population that merits consideration is patients with posttraumatic epilepsy (PTE). PTE is a common consequence of traumatic brain injury and accounts for about 20% of symptomatic epilepsy cases. These patients are often resistant to treatment with ASMs and may be unlikely to have a localizable lesion (Englot et al. 2012). VNS has also been used in severe epileptic encephalopathies such as CDKL5 deficiency disorder, suggesting that it is a generally safe and effective adjunct treatment (Lim et al. 2018).

It is interesting to note that as VNS use increases, revision surgeries have also increased (see Sect. 24.9), and although the rate of complications of VNS revision surgeries is acceptably low, it emphasizes the fact that indications for VNS today require better guidance. Based on a retrospective review of indications and complications of VNS revision surgery, Spindler emphasizes that VNS implantation should not be carried out until a reasonable pre-operative investigation and adequate discussion of all possible treatment options has been conducted. If a precise indication for VNS is present, the high likelihood of subsequent surgeries should be considered but not preclude recommendations for VNS (Spindler et al. 2021a).

Once the VNS option is discussed with the patient, there should be an extensive explanation about the cost of the device, the reduced possibility that the patient will be seizure-free, and all other risks and potential benefits, assuring a well-informed decision. Patients/caregivers should be informed that the antiepileptic effect is generally delayed after the procedure, as well as about the difficulty of removing the vagal electrode and the need to replace the battery after its useful life.

24.4 Technology

Since the first NeuroCybernetic Prosthesis (NCP, initial name of the VNS therapy) generator was approved, five generations of VNS System technology have been released. Apart from generator size, battery life, and software features, most



Fig. 24.2 Iterations of VNS generators



Fig. 24.3 The AspireSR® M106 generator provided the first responsive, closed-loop form of VNS Therapy using a CBSD algorithm

components have been conserved throughout the therapy’s evolution (Fig. 24.2). Healthcare providers today commonly encounter a range of single- and dual-pin generators (models 100, 101, 102, 102R, 103, 104, 105, 106, and 1000) and related programming systems (models 250 and 3000), all of which have their own subtle, but practical differences (Afra et al. 2021). The VNS Therapy System technology has undergone numerous hardware upgrades to reduce the implantable pulse generator’s size and weight, increase implantable lead durability, and optimize the generator’s circuitry to detect and process cardiac signals. Software improvements include increased communication speeds, automatic scheduled stimulation dose changes, the ability to perform cardiac-triggered stimulation, and the incorporation of event detection markers to inform clinical decision-making. The review of each of these changes is outside the scope of this review, but it should be kept in mind that device manuals should be carefully read and studied by the multidisciplinary team. The release of the AspireSR® M106 generator (2015) provided the first responsive, closed-loop form of VNS Therapy (Fig. 24.3). This optional automatic stimulation (AutoStim) Mode is delivered according to a CBSD algorithm. It has

been estimated that 82% of patients with epilepsy experience rapid heart rate increase associated with a seizure, especially in terms of generalized tonic-clonic seizures so heart rate increase is used as a proxy or biomarker of seizure activity. Ictal [tachycardia](#) occurs when hyper-excitation affects brain regions located in or directly connected to the mesial [temporal lobe](#), responsible for autonomic control of cardiac rhythm. Responsive VNS therapy combines open-loop stimulation that delivers a long-term neuromodulatory effect in order to reduce seizure frequency and -severity and automatic stimulation by CBSD in an attempt to terminate seizures and reduce seizure propagation (Boon et al. [2015](#)). Real-world use of the AutoStim Mode has been shown to increase VNS Therapy therapeutic efficacy in both pediatric and adult patients across varying epilepsy etiologies. Patients who elect to switch from a conventional VNS Therapy generator to a closed-loop AutoStim generator can experience additional therapeutic improvement upon generator replacement. New system features include a wireless programming wand, remote titration capability through Scheduled Programming, Day/Night Programming for patients who, for example, have diurnal fluctuations of seizure activity, and event detections that potentially serve as clinical biomarkers for risk of sudden unexpected death in epilepsy.

Another recent development that awaits consideration and the author has little experience with is non-invasive VNS (nVNS), which theoretically offers the advantage of avoiding the most common VNS-associated adverse events (Schulze-Bonhage [2017](#)). The primary advantage of the non-invasive based treatment is avoiding surgery and therefore avoiding implantation-associated adverse events such as infection and vocal cord paresis. Additionally, nVNS claims to limit stimulation-related adverse events by allowing greater customization of the stimulation paradigm. NEMOS (Cerbomed, Erlangen, Germany) is an external transcutaneous VNS available in Germany, Austria, Switzerland, and Italy (Ben-Menachem et al. [2015](#)). Testing out to response to this technology before committing to implantation in the selected group of patients might be an avenue of management.

24.5 Surgical Procedure

As mentioned, a review of different models and hardware changes is outside the scope of this chapter but the general surgical implantation technique, which should usually take 1–2 h, will be described generally (Fig. [24.4](#)). General anesthesia is preferred although, in principle, local anesthesia is feasible. It is often stated that the procedure can be performed by neurosurgeons, vascular surgeons, or ear, nose, and throat specialists, familiar with the surgical anatomy of the VN adjacent to the carotid artery. This is evidently correct, but it must always be considered that the implantation procedure must be preceded by a protocolized presurgical evaluation and followed up by the programming of the device in a comprehensive program that specializes in intractable seizures. The patient is positioned supine with a shoulder roll beneath the scapula to provide mild neck extension and the head rotated 30°

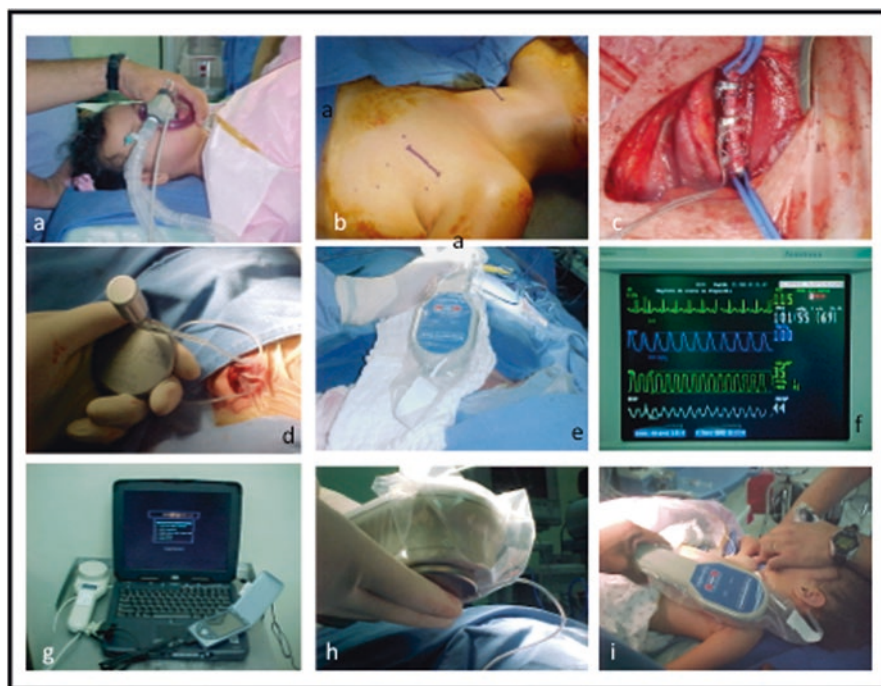


Fig. 24.4 Sequence of the surgical procedure: showing general anesthesia (a); positioning of the patient showing markers for cutaneous incisions (b); electrodes wrapped around the VN (c); with the hair pin resistor inserted into the receptacles for the lead connector pins, the telemetry wand interrogates the device from within a sterile sheath (d, e); monitoring of electrocardiographic and vital signs during the stimulation test (f); external programming equipment (g); final impedance test (h); and percutaneous tests (i)

toward the right. Prior to asepsis and antisepsis, sterile drapes are placed and a 3-cm horizontal incision in the lateral neck is made, from the internal border of the sternocleidomastoid muscle to the midline; the platysma muscle is divided vertically, and the investing layer of deep cervical fascia is opened along the anterior border of the sternocleidomastoid muscle, allowing it to be mobilized laterally to place a Weitlander retractor. The thyroid, trachea, esophagus, and laryngeal recurrent nerve contained in the pretracheal fascia are retracted in the bloc medially. The carotid sheath is incised with Metzenbaum scissors, and the deep aponeurosis is dissected to identify the jugular vein, the VN, and the common carotid artery. An automatic valve retractor is placed to contain the structures of the midline medially and the muscular mass laterally. The dissection of the VN, at least 3.5 cm in length, is carried out carefully with Metzenbaum scissors and DeBakey tweezers. In case of bleeding, a bipolar coagulator should be used. Next, a transversal 5-cm incision along the lateral border of the pectoralis major muscle and a subcutaneous pocket in the subcutaneous-muscle juncture is created to contain the generator. A tunneling tool is then used to create a subcutaneous tract between the two incisions. The field

is irrigated with physiological solution and antibiotics. Depending on the relative size of the exposed nerve, either a small or large helical electrode is selected. It should fit snugly without constricting the nerve. The lead (2.0 mm/0.08 in) should accommodate most nerves.

The lead connector pins are passed through the tunnel and emerge from the chest incision, while the helical electrodes remain exposed in the cervical region. The surgeon should grasp the suture tail at either end gently and apply each coil by stretching it over the nerve. The central turn of the unfurled coil is applied either obliquely or perpendicularly across or beneath the VN trunk and wrapped around it. The coil is then redirected parallel to the nerve as the remaining loops are applied proximal and distal to this midpoint. During these maneuvers, the generator is tested. With the hairpin resistor inserted into the receptacles for the lead connector pins, the telemetry wand interrogates the device from within a sterile sheath to measure the internal impedance. Once the generator passes the pre-implantation test, it is ready for insertion. The lead connector pins are inserted into the pulse generator and secured to their receptacles with setscrews, using the included hexagonal torque wrench. A 1-min lead test is performed at a frequency of 20 Hz with an output current of 1 mA and a pulse width of 500 μ s, during which the patient's vital signs and electrocardiographic changes are monitored. After the stimulation test, the generator is restored to its inactive status. The distal lead is secured to the fascia of the carotid sheath. Finally, the generator is retracted into the pocket and secured to the pectoralis fascia using a non-absorbable suture, using the suture hole contained within the epoxy resin holder. Wound closure proceeds in the standard multilayer fashion.

For generators with the AutoStim feature, the physical location of the device critically affects its ability to properly sense heartbeats. Therefore, care must be taken to follow the implant location selection outlined in the device's manual.

In children, a subpectoral technique for generator implantation has been described and should be considered given the increased soft tissue coverage, improved cosmesis, lower risk of tampering or trauma, and a comparable risk of infection (Bauman et al. 2006). The placement of the generator should preferably be above rib 4 or above, so the patient can have the maximum flexibility for MRI post-operatively.

The device is usually activated 2 weeks post-operatively, often at the first outpatient follow-up. Continuous electrical stimulation of the VN in animal models has been shown to produce fibrosis and ultimately failure of the nerve, so stimulation is provided in an intermittent manner.

24.6 Magnet Use

Patients or caregivers can pass a hand-held magnet over the implanted generator to initiate on-demand stimulation, typically when a seizure is anticipated or is in progress. Commonly the current delivered by magnet-induced activation is set slightly higher than the regular level. The magnet also lets the patient/caregiver deactivate

the device. When the magnet is subsequently removed, it reactivates at the previous settings. Boon et al. (Boon et al. 2001) described magnet used by 35 patients followed at a single center for an average of 35 months. Of the 21 patients who used the magnet, 7 reported no effect and 14 (67%) noted positive effects. Morris (2003) analyzed the data on magnet-activated VNS therapy from the double-blind, randomized E03 study (The Vagus Nerve Stimulation Study Group 1995) and the nonrandomized E04 study (Labar et al. 1999). This retrospective analysis of E03 and E04 data found that approximately half of the VNS therapy patients who used the magnet to activate stimulation gained some control over their seizures. About one-fifth of the patients in the treatment arm of the E03 study and the E04 study reported that they could abort seizures with the magnet. The E03 study analysis showed no correlation between the extent of magnet use and change in seizure frequency with programmed VNS therapy as measured during the acute phase of the trial. In a similar finding from the E04 trial, about two-thirds of the patients reporting magnet-activated improvement of more than 90% of their seizures were classified as non-responders to programmed VNS therapy because they experienced seizure frequency reductions of 50% or less. For these patients, magnet-activated, on-demand stimulation appears to have been VNS therapy's most important contribution to gaining a sense of control over their seizures. Achieving the best results from magnet-activated stimulation requires both appropriate VNS device settings and proper instruction in magnet use for patients and caregivers. The magnet itself has sustained changes over the different devices, the initial Block and Horseshoe magnets were replaced by the Cyber magnet that could be worn as a wristband or a clip attached to the belt. Magnets also differ in their reaction time to be able to stop stimulation but in general, magnet-activated, on-demand stimulation appears to have been an important contribution of VNS therapy to gaining a sense of control over seizures. Additional studies of programming settings for magnet-activated stimulation and analysis of quality-of-life aspects, such as increased sense of control or empowerment may provide a better understanding of this unique mode of delivering anti-seizure therapy.

24.7 Stimulation Protocols

Standard parameter settings, as determined from the clinical trials range from 20 to 30 Hz at a pulse width of 250–500 μ s and an output current of 0.25–3.5 mA for 30 s on time and 5 min off time. Initial stimulation is set at the low end of these ranges and slowly adjusted over time based on patient tolerance and response. The position of the fascicles and key fascicles needed for effective stimulation may vary among patients and depending on the size of the nerve; the electrode may not fully encircle it. If the fibers of interest are in the uncovered region, they may require more current for activation. Patients should be closely monitored during the dose adjustment phase, typically every 2–4 weeks for the first 2 months following implantation. Optimizing response can be done by increasing output current or modifying ON/

OFF times (duty cycles). Managing side effects can be done by decreasing signal frequency (from 30 Hz to 20 Hz), decreasing output current (by 0.25 mA), reducing pulse width (from 500 μ s to 250 μ s), or reducing output current by 0.25 mA. In clinical practice, migration toward 20 Hz from the original manufacturer setting of 30 Hz occurred in response to anecdotal reports of improved mood effects at 20 Hz, without impact on anti-convulsive effect. These findings were used to support the manufacturer's decision to change the factory setting for signal frequency from 30 Hz to 20 Hz with the introduction of the most recent [pulse generator](#). With the launch of the VNS Therapy System in the mid-1990s, there was a limited understanding of the dose-response profile of this device-based intervention. Physicians lack a biomarker of VN engagement that is reliably and easily measured in all patients, responds acutely to stimulation, and is readily deployable in most clinical settings, perhaps an equivalent of bioavailability and pharmacokinetics of pharmacotherapies, and have therefore relied more on a pragmatic approach of dose trial-ing. This has led to high inter-practice variability and frequent misalignment with practice guidelines and manufacturers' recommendations. A recent retrospective analysis (Fahoum et al. [2022](#)) using a generalized linear mixed model explored the relationship between key stimulation parameters (i.e., Output Current, Pulse Width, Signal Frequency, and Duty Cycle) and responder rate. A population-level target output current and duty cycle for VNS therapy for epilepsy was identified as 1.61 mA and 17.1% duty cycle. Patients with a shorter duration of epilepsy were identified to have a higher likelihood to respond to VNS therapy ($p < 0.001$). While patients who were on the therapy longer were more likely to respond to the therapy, the effect did not interact with the dosing settings, suggesting that patients who have been chronically underdosed may still benefit from achieving the target dose. However, this recommendation is based on retrospective analysis and over 90% of the database described outcomes from traditional VNS devices that did not have modern features like closed-loop stimulation functionality. In practice, once a patient responds to a tolerated stimulation dose, further parameter adjustments are performed only as clinically required. The best approach is to guide stimulation parameter titration on an individual basis. The output current should be the principal consideration when titrating patients to their individualized optimal dose, and individual patients may have optimal VNS output currents above or below a population-level target depending on their unique circumstances. Some centers have used "rapid cycling" VNS, which is to program the device with a higher duty cycle (definition varies from OFF-Time ≤ 1.8 min to a combination of ON-Time = 7 s and OFF-Time = 0.2–0.3 min) in case standard cycling is found to be ineffective. This technique was shown to be safe and potentially more effective than the standard cycling in pediatric patients (Kayyali et al. [2020](#)). Routine assessments of lead wire integrity and generator function are required in regular follow-up visits. Battery life, which depends on output and magnet use and the generator model, or for instance if the AutoStim feature is enabled in the later models, is likely to exceed 6 years even at higher output levels, after which the pulse generator will need to be replaced. The device should be checked regularly and an early replacement indicator or "near end of service" (NEOS) alert in the NCP and M102 series generators warns the clinician

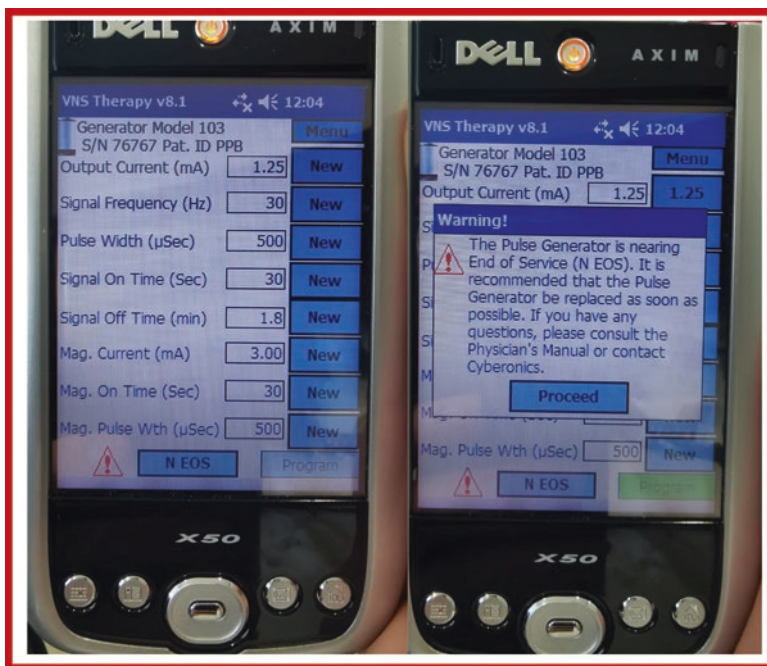


Fig. 24.5 The “near end of service” (NEOS) alert in the NCP and M102 series generators warns the clinician of impending battery exhaustion

of impending battery exhaustion (Fig. 24.5). For all other generator models, the remaining battery power is indicated by a battery icon on the Programmer’s home screen that represents the generator’s battery voltage level: At the NEOS level, it is recommended that the pulse generator be replaced as soon as possible. If the pulse generator is not replaced in a timely manner, it will eventually lose the ability to communicate with the programming software and poses the risk of reducing any therapeutic effect gained with the therapy. Finally, for NCP and M102 series generators, consideration should be made not to use frequencies of 5 Hz or less for long-term stimulation because these low frequencies have been shown to generate an electromagnetic trigger signal that results in excessive battery depletion.

24.8 Complications and Adverse Effects

Various complications and adverse effects have been well documented, although their incidence varies greatly in different reports, and in most cases, adverse effects decrease over time or can be resolved by changing stimulation parameters. Secondary stimulation related effects (viscerosensory symptoms, cough, hoarseness, dyspnea, dysphonia, dysphagia, neck, throat, and chin numbness) are usually

Table 24.1 Complications from a large (436 patients) single-center study (Elliott et al. 2011a, b)

Complication	Number (%)	
	Transient or Minor	Permanent or required revision or removal
<i>Neurological</i>	6 (1.4%)	12 (2.8%)
Hoarseness	6 (1.4%)	9 (2.3%)
Dysphagia	–	2 (0.5%)
Unilateral vocal cord paralysis)	–	1 (0.2%
<i>Non-neurological</i>	8 (1.8%)	11 (2.5%)
Neck/arm pain unrelated to duty cycle	2 (0.5%)	–
Neck pain related to duty cycle	3 (0.7%)	3 (0.7%)
Superficial infection (antibiotics)	2 (0.5%)	–
Deep infection (removal/revision)	–	7 (1.6%)
Seroma/hematoma requiring aspiration	1 (0.2%)	–
Pneumothorax	–	1 (0.2%)

Elliott et al. (2011a)

described as mild. Reported complications from a large (436 patients) single-center study are shown in Table 24.1 (Elliot et al. 2011a). Early clinical studies reported that if electrodes were placed below the cardiac branch of the VN, no cardiac effects would be manifested during stimulation. However, some ventricular asystole cases during impedance tests were reported (Ascanope et al. 1999) and according to the manufacturer’s database, 98 patients from 60,014 implantations developed asystole or bradycardia during implantation (Cyberonics data on file, May 2008). The main reason for this phenomenon is the lack of dissection of the cardiac branch during the procedure or the lack of anatomic knowledge

Surgical infection is the most described complication, although the rates of 2–20% and 0.7–7.7% (Air et al. 2009; Benifla et al. 2006; Elliot et al. 2011a, b) in adults and children, respectively, have varied considerably. It is interesting to note that larger series have generally reported lower infection rates, i.e., 0.7% infection rate in the series reported by Elliott et al. (2011a, b), 2.7% in the series by Thompson et al. (2012), and the author’s own series of 100 consecutive cases with a 0% infection rate, which suggests that proper and careful surgical technique, surgical volume, and experience contribute to beneficial outcomes for VNS therapy.

The possibility of tissue damage has been a concern; however, stimulation parameters have not been associated with nerve damage. Diathermy (shortwave, microwave, ultrasound) should not be used on VNS therapy patients. It has been demonstrated that cellular phones and security systems at airports and commercial centers do not affect pulse generators or electrodes. There is, however, some concern regarding limitations of new generation MRI, including patients in whom the device has been removed but the wire remains. The potential risks of performing MRI on patients with an implanted VNS include heating effects, especially of the stimulation electrodes, inadvertent resetting of the device or magnet activation,

image distortion and artifacts, magnetic field interactions, and device malfunction or damage. If an MRI scan must be performed, VNS output should be set to zero beforehand and reset afterward. VNS is approved in MRI scanning using only transmit-and-receive type head coils at both 1.5 and 3T field strength. Some modern head coils are of the phased-array type, which should not be used. In practice, good diagnostic quality brain scanning can be achieved if appropriate precautions are in place, however, body or extremity imaging (receive only coils) and experimental brain protocols are discouraged, even if the generator has been explanted and only the wire remains. It is advisable to consult the device manufacturer if there is any doubt.

24.9 Device Revisions and Removals

As the use of VNS devices increases, and patients meet longer follow-up periods, revisions and removals have become more of an issue to be considered. In the retrospective review of a prospectively created database of 436 consecutive patients (Elliott et al. 2011a, b) who underwent VNS for DRE, 129 patients (29.6%) underwent a total of 155 VNS revisions after primary implantation. The most common indication for revision was generator power depletion, which occurred at a mean of 47.7 ± 18.9 months following implantation or last generator change (range: 23–106 months). Lead fracture occurred in 20 devices and presented with delayed neck pain in synchrony with the duty cycle in 17 cases or by loss of device efficacy in 3. Seventy-four patients (17.0%) underwent device removal following primary insertion at a mean of 40.4 ± 30.6 months. Indications for VNS device removal were non-efficacy/worse seizures in 32, MRI for possible or planned IES or other MRI indications in 31, infection in 7, ASM success in 3, and vocal cord paralysis in 1 case. There were no complications during device removal. In a more recent study of revision surgery of 90 VNS procedures 54.4% were revision surgeries (Spindler 2021). The vast majority was due to the depletion of the battery. The entire system was explanted in 15 patients, due to no beneficial effect detected ($n = 4$), due to irritating side effects ($n = 4$), and so further diagnostics could be carried out ($n = 7$). Interestingly in three of the patients who underwent further diagnostics, resective epilepsy surgery was performed. Surgical complications occurred in 8.2%.

24.10 Results

24.10.1 Seizure Reduction

The meta-analysis performed by Englot et al. (2011) through a PubMed query for all articles in the English literature published up to November 2010 using the search terms alone and in combination: “seizure,” “epilepsy,” “vagus,” “vagal,” “nerve,”

“stimulation,” “stimulator,” and “surgery” identified 74 clinical studies of VNS in epilepsy including 3321 patients. These studies consisted of 3 blinded, randomized controlled trials (Class I evidence); 2 nonblinded, randomized controlled trials (Class II evidence); 10 studies reporting prospective data (Class III evidence); and numerous retrospective studies. Among prospective studies, seizure reduction rates were 17–55% after 3–64 months of VNS therapy, with 21–50% of patients experiencing $\geq 50\%$ decrease in seizure frequency. Across all studies, VNS reduced seizure frequency by approximately 45%, although the rate of seizure reduction increased from 36% at the 3- to 12-month follow-up to 51% after >1 year of therapy. In examining outcomes using the Engel outcome scale, the authors found that $\approx 50\%$ of patients attained a clinically significant reduction in seizure frequency $\geq 50\%$, with about 12% experiencing $\geq 90\%$ decrease in seizures. Overall, VNS predicted $\geq 50\%$ reduction in seizures with a main effects Odds Ratio (OR) of 1.83 (95% CI 1.80–1.86). A summary of class of data, number of patients studied, minimum follow-up, percentage of patients achieving seizure freedom, and responder rate are given in Table 24.2. Approximately 25% of the patients in the published literature did not receive a measurable clinical benefit and complete seizure freedom was rarely ($<5\%$) attained. The authors also stratified outcomes by patient age and seizure type and observed that children experienced a slightly better outcome than adults (55% vs. 50% reduction in seizures, respectively), with patients younger than 6 years old achieving a 62% decrease in seizure frequency. Furthermore, patients with generalized epilepsy received increased benefit compared with those with partial seizures (58% vs. 43% reduction in seizures). Caution must be used in interpreting these results, as data on age and type of seizure is frequently missing in source studies. Therefore, while the data are insufficient to determine if VNS truly conveys increased benefit in children and in patients with generalized epilepsy, available data do suggest that both patient groups may receive benefit from VNS therapy despite initial exclusion during device approval. The one aspect that has already been mentioned and is consistent in most studies is that the improvement in terms of seizure reduction tends to increase over time. The long-term improvement over time in the open label and a post-approval study is shown in Fig. 24.6. On the other hand, there have been studies to support the earlier implantation of VNS therapy to increase the likelihood of success (Soleman et al. 2018).

The efficacy of the AutoStim mode, which is sometimes referred to as superior to the earlier models, was studied in a multisite trial in the United States (E-37), a prospective unblinded study in 20 patients with medically refractory partial onset seizures and history of ictal tachycardia. At 12 months, quality of life (QoL) and seizure severity scores improved with a responder rate of 50%. During an inpatient observation period, about 43% of all seizures occurred with at least a 20% increase in heart rate compared to baseline heart rate and complex partial seizures were most likely to be associated with higher heart rate increases. Extra stimulations triggered by ictal tachycardia did not significantly affect battery life, with measured duty cycles increasing from 11% to 16% with AutoStim activated (Fisher et al. 2016). The other multisite trial was performed in Europe (E-36) where responder rate at 12 months was reported as 29.6% (Boon et al. 2015). Albeit being a retrospective

Table 24.2 Summary of class of data, patients studied, minimum follow-up, patients achieving seizure freedom, and responders rate in publications investigating outcome in VNS

Author/year	Evidence class	No Patients	Minimal Follow-up (months)	% of patients	
				Seizure free	Responders (at follow-up when indicated)
Ben-Menachem et al. (1994)	I	67	3.5	–	38.7
Handforth et al. (1998)	I	196 ^a	3	–	–
George et al. (1994)	II	67	16	–	–
Ben-Menachem et al. (1995)	II	16	9	–	–
Salinsky et al. (1996)	II	100	24	–	18.4
Ben-Menachem et al. (1999)	II	64	3	–	40.4
Vonck et al. (1999)	II	15	12	27	67
Sirven et al. (2000)	II	45	3	–	67
Ardesch et al. (2007)	II	19	12	–	36.8
Janszky et al. (2005)	III	47	12	13	–
Murphy (1999)	III	60	3	–	–
Chavel et al. (2003)	III	29	24	–	61
Murphy et al. (2003)	III	96	6	–	45
Vonck et al. (2004)	III	118	6	7 ^b	50
Labar (2004)	III	269	3	6.3	56.9 (12 months)
Benifla et al. (2006)	III	41	6	–	3.8
Saneto et al. (2006)	III	43	9	–	51
De Herdt et al. (2007)	III	138	12	9	59 (44 months)
Montavont et al. (2007)	III	50	21.6	0	61 (24 months)
Ghaemi et al. (2010)	III	144	24	6.9	61.8
Alonso-Vanegas et al. (2010)	III	35	12	5.7	74.3 (12 months)
Elliott et al. (2011a)	III	436	3	–	63.7 (59 months)

(continued)

Table 24.2 (continued)

Author/year	Evidence class	No Patients	Minimal Follow-up (months)	% of patients	
				Seizure free	Responders (at follow-up when indicated)
Elliot et al. (2011b)	III	141	3	–	64.8
Orosz et al. (2014)	III	347	6	6.7	43.8 (24 months)
Kawai et al. (2017)	III	362	3	–	58.8 (36 months)
Chrastina et al. (2018)	III	74	12	0	77.8 (17 years)
Soleman et al. (2018)	III	Early group+14	12	14.3	64.3
		Late group+31		3.2	41.9
Hamilton et al. (2018)	III	51 AspireSR®	3	0	59 (13 months)
		62 Reinsertions with AspireSR®	3	0	71 (21 months)
Xu et al. (2022)	III	76	6	6.6	68.4 (4 years)

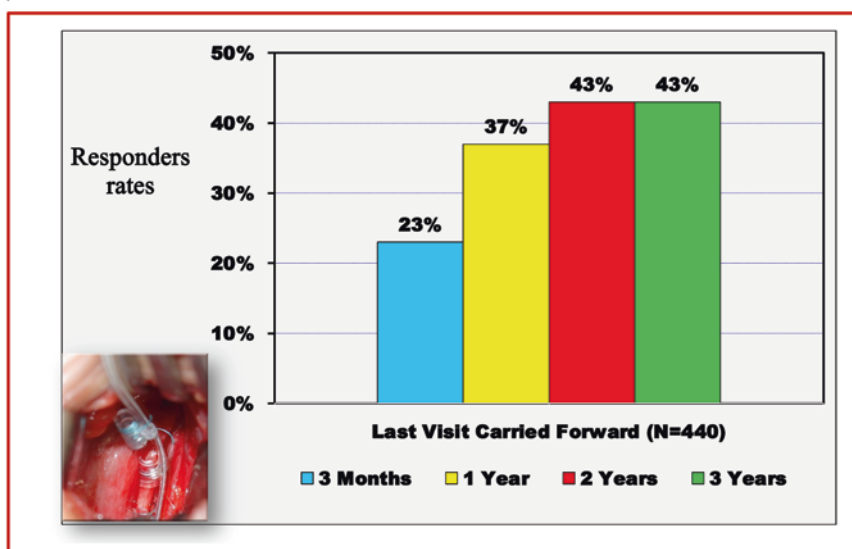
– not reported
+ Early group = ≤5 years of age Late group = >5 years of age
*Value reflects the intent-to-treat population
^bFree of seizures with impaired consciousness

analysis and not randomized or biased, a study positing that the response to closed-loop VNS device would be better showed that approximately 70% of patients with existing VNS insertions could have significant additional benefits from cardiac-based seizure detection and closed-loop stimulation from the AspireSR device. For new insertions, the AspireSR device has efficacy in 59% of patients (Hamilton et al. 2018). Spindler et al. have concurred that closed-looped VNS therapy is well tolerated without added adverse effects and shows good sensitivity given that patients who report more seizures per week have higher percentages of AutoStim. An extremely high number of AutoStim despite relatively few seizures indicates poor specificity and although a promising feature in the development of a patient-tailored therapy, the CBSD algorithm should be revised to provide better specificity (Spindler et al. 2021a, b).

24.10.2 *Quality of Life (QoL) and Other Neuropsychological Variables*

Preliminary studies reported additional effects of VNS on neuropsychological variables, such as mood, alertness, memory, and postictal recovery periods. Although different mood scales were used, several studies have demonstrated mood improvements after treatment with VNS (Elger et al. 2000; Aldenkamp et al. 2001). None of

a)



b)

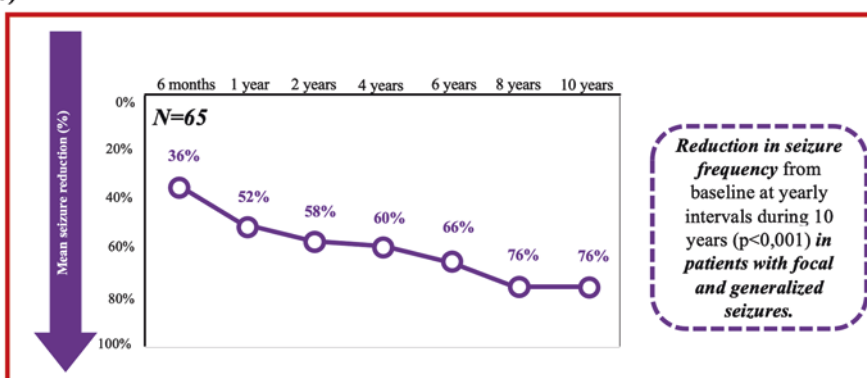


Fig. 24.6 Improvement over time in primary seizure outcome measure. (a) of the open label, long term efficacy and safety/tolerability study (E01-E05). Adapted from Morris GL and Muller WM, *Neurology*. 1999;53(7):1731–1735. (b) 10-year study period in a retrospective review of a prospectively created database of 436 consecutive patients. Adapted from Elliott RE., et al. *Epilepsy Behav*. 2011;20:478–83

these studies have found an association between seizure reduction and mood improvement. This may indicate specific additional effects of VNS on mood, which may be independent of improved seizure control. This treatment effect became relevant for the treatment of mood disorders in general, and after a randomized controlled trial and several clinical trial data the FDA approved VNS as treatment for therapy-resistant depression in 2005 (FDA approvals, 2005). The effects of VNS on QoL, which is more difficult to assess quantitatively, remain controversial. Some studies (McLachlan et al. 2003) have reported increased health-related QoL,

whereas McGlone et al. have found no change in QoL (McGlone et al. 2008). Klinkenberg et al. (2012a) studied 41 patients with refractory epilepsy and found significant improvements for both mood and QoL after 6 months of VNS, based on the results in the Profile of Mood States (POMS) and QoLIE-89 questionnaires ($p < 0.05$). There was no significant change in cognition. The mean percentage change in seizure frequency was -9.0% , while 20% of the patients achieved a seizure frequency reduction of 50% or more. No significant correlation was found between changes in seizure frequency and improvements in mood or QoL. Concerning cognition, both Dodrill (Dodrill and Morris 2001) and Hoppe et al. (2001) report no change in cognition in VNS therapy. Aldenkamp et al. (2002) have demonstrated an improvement in mental age in children independent of seizure control. A possible explanation for this improvement in functioning might be the improved quality of sleep (Hallbook et al. 2005). Although statistically significant, the IQ increase observed in the high-level stimulation group in the controlled trial by Klinkenberg (Klinkenberg et al. 2012a, b) is considered too modest to be clinically relevant and in fact could no longer be demonstrated at the end of the follow-up period. One may conclude that VNS at least does not have any negative effects on cognition, in contrast to some ASMs, especially in the case of polytherapy.

Many studies have shown that irrespective of seizure outcomes, patients, parents, and caregivers report an improvement, even if subjective, in overall quality of life as a conglomerate of ASM burden, mood, memory, alertness, and reduction of postictal duration (Fig. 24.7). Soleman et al. compared seizure outcome and quality of life

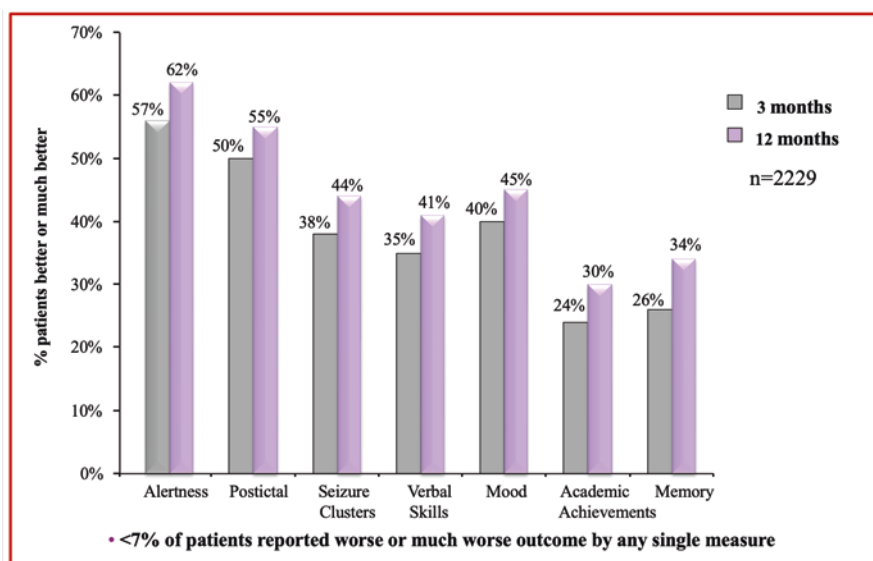


Fig. 24.7 Improvements in a conglomerate of measures of quality of life (QoL) over time. (1) Data on file. VNS Therapy patient outcome registry. Cyberonics, Inc., Houston; 2003. (2) Ergene E., et al. *Epilepsy Behav* 2001;2:284–7. (3) McLachlan RS, et al. *Eur Neurol* 2003;50:16–9. (4) Harden CL., et al. *Epilepsy Behav* 2000;1:93–9. (5) Hoppe C., et al. *Epilepsy Behav* 2001;2:335–42

after early (≤ 5 years of age) and late (over years of age) implantation of VNS in children. Reduction of seizure frequency, responder rate, and reduction of ASM were comparable in both groups, but the measures of quality of life (total and cognitive), Pediatric Quality of Life (PEDSQLTM) Core questionnaires, and caregiver impression (CGI) scale between preoperative and follow-up was significantly higher in the early implantation group (Soleman et al. 2018). This warrants the consideration of VNS even in toddlers and children under the age of 5 years and definitely adds to the overall growing evidence that earlier intervention in DRE is of paramount importance.

24.10.3 ASMs

The possibility of reducing ASMs in the postsurgical period is a common and often controverted topic in every surgical option for intractable epilepsy, and it has been mentioned as a potential advantage of VNS, which would be particularly beneficial in the pediatric population. However, in the authors' experience (Alonso-Vanegas et al. 2010) as well as in reports of long-term studies, it is generally not possible to reduce the number of ASMs, though a reduction in dosage can often be used during some periods. In a study Majkowska-Zwolińska et al. (2012) in 57 children and adolescents the average number of ASMs taken over time decreased from 2.71 drugs per subject at 6 months to 2.27 drugs per subject at 48 months. This modest decrease is consistent with the results reported in other studies (De Herdt et al. 2007; Vonck et al. 2004) but it is noteworthy that two-thirds of children receiving benzodiazepines at baseline can withdraw from the drugs completely. This is important from a clinical point of view since chronic use of these drugs is associated with cognitive impairment, sedation, and tolerance. Reduction in the number of ASMs has generally only been found in small series (Hornig et al. 1997; Shahwan et al. 2009). It has proven difficult to evaluate the impact that changes in ASM regimens have on seizure frequency in the setting of VNS. In fact, as stated by Elliott et al. (2011a; b), the increase in VNS efficacy over time may be due to alteration in device parameters, changes in ASM regimen, or an undefined, synergistic effect of both.

24.11 Cost-Effectiveness

Several studies have reported the clinical and economic benefits of VNS. A study in Sweden reported an annual cost savings of \$3000 when comparing 18 months before and after VNS implantation among 43 patients, stating that the purchase price of a VN stimulator can be absorbed in 2–3 years (Ben-Menachem et al. 2002). Another study reported that the average annual direct medical costs decreased from \$4826 to \$2496 for 25 patients who underwent VNS in Belgium (Boon et al. 2002).

The cost estimates in both studies were reported in 1999 US dollars. In 2007, the average quarterly resource utilization for 12 months before implantation was compared with that of 48 months after implantation in 138 patients treated in the United States, and the investigators found that the use of healthcare resources, such as emergency room and outpatient visits, decreased after implantation (Bernstein et al. 2007). The first study (Helmers et al. 2011) to evaluate the long-term impact of VNS on resource utilization, epilepsy-related clinical events, and costs simultaneously in a very large cohort of 1655 patients found that average quarterly health care resource utilization (overall and seizure-related) decreased in the post-VNS period versus the pre-VNS period, even after adjusting for potential confounding factors. Hospitalizations decreased post-VNS compared with pre-VNS (adjusted IRR = 0.59, $p < 0.001$). Grand mal status events decreased post-VNS compared with pre-VNS (adjusted IRR = 0.79, $p < 0.001$). Average total health care costs were lower post-VNS than pre-VNS (\$18,550 vs. \$19,945 quarterly, $P < 0.001$). The average cost in the first quarter after implantation, including the cost of the device and implantation, was high at \$42,540 per patient per quarter, but this cost was outweighed at about 1.5 years postimplantation, producing net healthcare cost savings. In a review of routinely collected hospital data in England on the impact of VNS it was concluded that VNS is associated with increased outpatient resource utilization and decreased inpatient admissions, with a reduction in long-term epilepsy-related medical costs post-implantation (Camp et al. 2015). Naturally, it must be kept in mind that conclusions from cost-effectiveness studies can often not be generalized to broader populations, and different healthcare systems; likely different subsets of patients (including different epileptic syndromes, public or private healthcare systems) derive differential economic benefits from VNS.

24.12 Prognostic Factors and Future Directions

Several studies have tried to identify a profile of responders. The first prospective randomized active-controlled trial in children evaluating the effects of VNS frequency, comparing low versus high stimulation parameters (Klinkenberg et al. 2012a, b) found a trend toward a correlation between age at onset and response to VNS in line with the previous results of Patwardhan et al. (2000). According to Janszky et al. (2005), the absence of bilateral interictal epileptic discharges and the presence of malformation of cortical development were factors predicting a favorable outcome. In this randomized trial, seven out of nine participants in whom 50% or more seizure frequency reduction was achieved had bilateral interictal epileptic discharges compared with 18 out of 25 non-responders. However, only one of the 8 participants with malformation of cortical development had a 50% or more seizure frequency reduction. Callosotomy before VNS treatment has been associated with a positive response; this was not the case in one participant in the randomized trial. The role of these two palliative surgeries in LGS, namely, callosotomy and VNS has been explored in some series (Rolston et al. 2015) and a meta-analysis of

published data, showing that CC had a significantly better outcome than VNS for >50% atonic seizure reduction (80.0% [67.0–90.0%] vs. 54.1% [32.1–75.4%], $p < 0.05$) and for >75% atonic seizure reduction (70.0% [48.05–87.0%] vs. 26.3% [5.8–54.7%], $p < 0.05$). All other seizure types, as well as a total number of seizures, showed no statistically significant difference between VNS and CC (Lancman et al. 2013).

Other factors, presumably associated with a better response, have also proven controversial. For instance, in the meta-analysis by Englot et al. (2011) the greatest benefit from VNS was seen in patients with posttraumatic epilepsy (79% reduction in seizures) and with tuberous sclerosis (68% decrease in seizures), while individuals with an unknown or idiopathic epilepsy etiology experienced 51% fewer seizures, and patients suffering from Lennox-Gastaut syndrome or other epileptic encephalopathies had a 48% decrease in seizures, but only 517 of 3321 patients from the literature could be disaggregated by epilepsy etiology. There has been a consensus that shorter epilepsy duration and longer time on therapy are associated with improved patient outcomes (Fahoum et al. 2022). Interestingly, a shorter duration of epilepsy is associated with improved response probability, despite younger patients being less likely responders. A growing body of evidence supports earlier intervention with VNS to achieve the best outcomes with the therapy. Some answers might be available through a currently ongoing international, multicenter, prospective, observational, all-comers, post-market registry (CORE-VNS) with analysis endpoints including seizure frequency (average number of events per month), seizure severity (individual-rated categorical outcome including very mild, mild, moderate, severe or very severe) as well as non-seizure outcomes such as adverse events, use of antiseizure medications, use of other non-pharmacological therapies, quality of life, validated measures of quality of sleep (Pittsburgh Sleep Quality Index or Children's Sleep Habit Questionnaire) and healthcare resource utilization (Sen et al. 2021).

As mentioned in the section on MOA, different studies have focused on particular connectivity patterns or quantitative symmetry measures that seem to discriminate between responders and non-responders (Zhu et al. 2020; Yu et al. 2018; de Vos et al. 2011; Carron et al. 2022). These studies need to be extended and validated to create better models of predictive outcomes.

The emerging, yet challenging, trend of creating special neuromodulation clinics within comprehensive epilepsy centers is also worth systemizing to allow for efficient management of resources, scheduling field engineers, and specific training of multidisciplinary teams. The extended use of VNS therapy worldwide is promising but, in the scenario, where comprehensive centers treating DRE still lack systemizing guidelines and surgical options are underutilized or even in decline, it should also serve as a cautionary tale to better define the exact role of this neuromodulations therapy in providing the best clinical practice for patients with DRE. It should be emphasized that open surgical resection remains the gold standard treatment for DRE. VNS and other non-resective procedures have not replaced the need for resection, though there is hope that these surgical options can increase the number of patients who receive treatment for this devastating disorder.

24.13 Conclusion

As most articles evaluating the effect of VNS on intractable seizures in the current literature conclude, VNS is a safe, feasible, and well-tolerated option, with minimal side effects and complications, and improvement in seizure frequency and severity that often increase over time in selected groups of children and adults with partial or generalized seizures. Pending the still elusive precision in the understanding of MOA, factors that can predict a good response, and stimulation parameters/protocols that can be applied according to measurable clinical evidence and refinement of event markers, its use in a comprehensive epilepsy program is still a function of the multidisciplinary team's experience and patient-based judgment. An exhaustive presurgical evaluation of DRE patients that excludes resective surgical options has a higher probability of seizure freedom, while only then suggesting VNS with detailed regard to expense and risks weighted against potential improvements in seizures and quality of life is of paramount importance.

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Chapter 25

Noninvasive Brain Stimulation as a Potential Therapeutic Procedure in Drug-Resistant Epilepsy



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Abstract About only 5% of all patients with drug-resistant epilepsy can be considered candidates for surgery. Consequently, there is an enhancing interest in studying neuromodulatory techniques such as vagus nerve stimulation, deep brain stimulation, and noninvasive brain stimulation (NIBS) as potential therapeutic alternatives for patients who are *not* eligible for epilepsy surgery. *In this respect*, repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS) are the most commonly used NIBS methods. This chapter reviews the potential clinical use of NIBS in drug-resistant epilepsy therapy. After introducing some basic principles as well as safety and tolerability aspects, NIBS results in epilepsies are summarized. Next, some NIBS effects on functional connectivity using neurofunctional imaging are discussed. It is concluded that both rTMS and tDCS could be effective as adjuvant therapeutic approaches for patients with drug-resistant epilepsy who are *not* eligible for epilepsy surgery. However, further multi-center clinical studies would be of great help to clarify the effects of these strategies when combined with antiseizure medication, measures of outcome assessment, and eligibility criteria of patients, including novel methods such as personalized stimulation protocols based on computational modeling.

Keywords Drug-resistant epilepsies · Repetitive transcranial magnetic stimulation (rTMS) · Transcranial direct current stimulation (tDCS) · Functional connectivity

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25.1 Introduction

Epilepsy affects over 50 million people worldwide, and about 30% of these patients have resistance to treatment with antiseizure medications (ASMs) (Kuzan-Fischer et al. 2020). Namely, drug resistance or pharmacoresistant epilepsy is a health problem with a significant burden in comorbidity and an increased risk of premature death (Brodie 2005; Casadei et al. 2020; Ryvlin et al. 2011). Therefore, efforts to improve therapeutic options, both pharmacological and nonpharmacological treatments, which are currently in the pipeline, have as their main objective the best possible control of seizures (Abdullahi et al. 2022; Bex et al. 2022; Hilz 2022; Riva et al. 2021).

Despite the development of new and safer ASMs, there is no solid evidence to indicate the improvement of the efficacy of these new drugs in patients with drug-resistant epilepsy (Brodie 2005; Enia et al. 2021). In fact, it is known that, when two ASMs fail controlling seizures, the association of another one could have a success less than 5% (Perucca et al. 2011; Wiebe 2004; Wirrell 2013).

Although epilepsy surgery is not the first line of treatment, it is considered a therapeutic option in patients with drug-resistant epilepsy (DRE). It has been shown that surgical resection allows control of epileptic seizures in about 40–80% of patients who fulfill criteria for epilepsy surgery (60–80% in temporal lobe epilepsy, and 40–60% in extratemporal lobe epilepsy) (Morales Chacón et al. 2018, 2021b). Even so, there is a considerable number of patients who still present seizures after epilepsy surgery. Thus, there is a growing interest in applying neuromodulatory techniques such as vagus nerve stimulation, deep brain stimulation, and noninvasive brain stimulation (NIBS) as a potential strategy to control DRE (Brodie 2005; Hachem et al. 2019; Kwon et al. 2018; Watrous et al. 2015).

At present, repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS) are the most popular noninvasive brain stimulation (NIBS) technologies. These methods use electrical fields generated noninvasively in the brain to long-lastingly modulate the excitability/activity of brain regions contributing to relevant processes (Sanches et al. 2020). Originally, pulsed transcranial magnetic stimulation (TMS) was used for research and functional diagnosis exploring corticospinal pathways. In the 90s, Pascual-Leone et al. found evidence supporting its potential therapeutic use (Pascual-Leone et al. 1999). Likewise, the development of TMS and rTMS allowed the rediscovery of tDCS and its use with therapeutic purposes. However, it was not until the end of the 90s and the early 2000s that tDCS gained attention in the scientific community, resulting in a vast increase in the number of studies examining its potential clinical applications (Been et al. 2007; DeMarse and Carney 2014; Gómez et al. 2017, 2014; Nitsche et al. 2012; San-Juan et al. 2015).

Equally important, noninvasive brain stimulation (NIBS) has been seen common in rehabilitation settings for the treatment of stroke, spinal cord injury, traumatic brain injury, and multiple sclerosis, as well as for some diagnostic neurophysiological measurements (Kesikburun 2022). Correspondingly, neurological disorders

such as epilepsy, Parkinson's disease, Alzheimer's disease, and depression are conditions that can benefit from these emerging technologies (Eastin and Lopez-Gonzalez 2017).

There are currently some studies suggesting that both methodologies could be effective as adjuvant therapeutic approaches to control pharmacoresistant epilepsy in patients (Carvalho et al. 2018; Chan et al. 2018; Izadi et al. 2018; Lin et al. 2018; Ng et al. 2018). Explicitly, specific modulatory effects can be obtained with different NIBS techniques, improving activity in excitatory networks or increasing intracortical inhibition, and then, reducing the cortical excitability (Chervyakov et al. 2015; Muller-Dahlhaus and Vlachos 2013; Roche et al. 2015). Thus, this chapter will review the potential clinical use of NIBS in drug-resistant epilepsy therapy.

25.2 Non-invasive Brain Stimulation: Basic Principles and Protocols

Even if rTMS and tDCS share effects at the physiological level, their physical properties are not the same. These noninvasive techniques are applied directly through electrodes or magnetic fields on the scalp of the patient to produce electrical currents for the stimulation of brain cells (Camacho-Conde et al. 2022).

NIBS application may result in modulatory cortical inhibition/excitation balance with behavioral and physiological consequences that outlast the stimulation duration. Indeed, these effects may well last weeks or months when rTMS and tDCS are repetitively applied (He et al. 2020; Sever et al. 2022). Based on this information, NIBS might be useful as potential therapy for neurological and psychiatric disease such as pain, movement disorders, stroke, amyotrophic lateral sclerosis, multiple sclerosis, epilepsy, consciousness disorders, tinnitus, depression, anxiety disorders, obsessive-compulsive disorder, schizophrenia, craving/addiction, and conversion (Elyamany et al. 2021; Sanches et al. 2020).

25.2.1 Repetitive Transcranial Magnetic Stimulation

TMS is a neuromodulation technique that uses large transient magnetic fields to induce focal electrical fields in a specific brain area, and the availability of sophisticated equipment has made it possible to employ repetitive TMS (rTMS). The effects of rTMS vary depending on the shape of the coil (figure of eight, H coil, double cone coil), pacing pattern (high frequency, low frequency, theta-burst), and stimulation site (Camacho-Conde et al. 2022; Fregni et al. 2006a).

In addition, the effects induced by rTMS correlate with long-term depression (LTD) and long-term potentiation (LTP), two forms of synaptic plasticity elicited in animal models of cortical circuitry by low- and high-frequency electrical

stimulation, respectively (Bliss and Cooke 2011). Besides, rTMS applied at low frequency may exert antiepileptic effects by inducing LTD whereas at high-frequency stimulation, it may facilitate proconvulsant effects (Ziemann et al. 2015). Nevertheless, both phenomena by themselves are insufficient to elucidate the early and long-term changes that take place after short NIBS episodes. Other mechanisms, including enhancement of GABAergic inhibition (Pascual-Leone et al. 1999), may also be involved in the anticonvulsant effects caused by rTMS.

Previous studies using preclinical models indicate that rTMS applied at low frequency (0.5 Hz) reduces the occurrence of status epilepticus and increases the latency of pentylenetetrazole-induced seizures (Akamatsu et al. 2001). It has also been demonstrated in hippocampal and neocortical rat slices that low-frequency (1 Hz) electrical stimulation is able to prevent interictal epileptic discharges and epilepsy-like events in an intensity-frequency and distance-dependent manner. These effects persist after the end of stimulation and are NMDA-receptor dependent, thus indicating that LTD-inducing protocols might have antiepileptic properties (Lanza et al. 2022; Lefaucheur et al. 2014).

The findings reported in animal models are consistent with the potential therapeutic use of LF-rTMS in patients with epilepsy (Ben-Menachem and French 2005; Fregni et al. 2006b). However, further studies are required to analyze the effects of rTMS in experimental models of drug-resistant seizures.

Even though underlying mechanisms of the therapeutic outcomes of rTMS application have not been fully explained, rTMS can induce changes in cerebral blood flow, oxygen consumption, cortical activity, and release of neurotransmitters. As a result, it has been argued that these functional changes might be associated with positive clinical results (Camacho-Conde et al. 2022; George et al. 2003).

25.2.2 Transcranial Direct Current Stimulation (tDCS)

Transcranial direct current stimulation (tDCS) represents a re-emerging noninvasive brain stimulation technique that has been used in animal models and human trials aimed to elucidate neurophysiology and behavior interactions (Sudbrack-Oliveira et al. 2021). When using tDCS, continuous but low-intensity current is applied through electrodes (anode and cathode) placed on the scalp. High-definition tDCS (HD-tDCS) is a variant of this technique, and, in contrast to tDCS where distribution of electrical current in a target area is relatively diffused, HD-tDCS devices are used to increase focal stimulation of a target area. In comparison with rTMS, tDCS is not as powerful and generates weak stimulus; however, it is relatively easy to use and transport, lot less costly, and it has low incidence of side effects (He et al. 2020).

It has been shown that tDCS modulates spontaneous neuronal activity through changes in the resting membrane potential. Moreover, the effect of tDCS varies according to the type of current (direct, alternating, pulsed, and random noise), polarity (anodal or cathodal), current intensity, and stimulation site (Guleyupoglu et al. 2013).

Concerning changes of tDCS in the motor cortex excitability, previous research findings have shown that they are polarity dependent, i.e., anodal tDCS increases motor cortex excitability, whereas cathodal tDCS decreases it. Equally, anodal tDCS increases motor-evoked potential amplitude by about 40%, while cathodal tDCS decreases it (Nitsche et al. 2012; Sudbrack-Oliveira et al. 2021).

A number of studies corroborate that tDCS effects are mediated by D2 and NMDA receptors, regardless of the polarity used (Auvichayapat and Auvichayapat 2011; Basavaraju et al. 2019). For example, using magnetic resonance spectroscopy, Stagg et al. 2010, observed that anodal tDCS decreased GABA brain concentration, with no changes in glutamate concentration, whereas cathodal tDCS reduced both glutamate and GABA concentrations (Stagg et al. 2010). Other tDCS effects include changes in dopamine, acetylcholine, and serotonin (Beuthien-Baumann et al. 2005; Cirillo et al. 2017).

Furthermore, preliminary experimental studies indicate a multifaceted scenario potentially relevant to the therapeutic effects of NIBS, including gene activation/regulation, de novo protein expression, morphological changes, alterations in intrinsic firing properties, and modified network properties resulting from changed inhibition, homeostatic processes, and glial function (Cirillo et al. 2017).

25.3 Safety and Tolerability of Noninvasive Brain Stimulation in Patients with Drug-Resistant Epilepsy

During the past two decades, there has been an increasing use of NIBS techniques to treat drug-resistant epilepsy in patients (Acerbo et al. 2022; Alicart et al. 2021; Bermpohl et al. 2006; Nourski et al. 2015). Nevertheless, it is clear that further research should be done to support the effectiveness of these therapeutic strategies. An important issue is that they induce few adverse effects (Been et al. 2007; Begemann et al. 2020; Caldwell et al. 2019). It has commonly been assumed that adverse effects are more associated with rTMS than with tDCS, comprising transient headaches and scalp discomfort as a consequence of activation of scalp pericranial muscles. It has also been demonstrated that it induces tenderness and neck pain. Conversely, TMS seldom induces seizures. Unusual adverse effects also involve syncope, fainting, nausea, vomiting, auditory change, and hypomania. (Muller et al. 2012; Pereira et al. 2016; Zewdie et al. 2020).

In general, the most common adverse effects induced by tDCS encompass local pain, tingling, itching or skin irritation on the stimulation area, fatigue, drowsiness and headache (Brandt et al. 1997; Cortes et al. 2017; Ille et al. 2016; Zewdie et al. 2020).

It is a widely held view that the most feared adverse effect induced by NIBS in patients with epilepsy is the induction of seizures. In this respect, the occurrence of focal seizures was described as a result of applying tDCS with the anode placed on the paracentral region (1.2 mA, 20 min), in a four-year patient with history of

epileptic spasms 2 years prior to the intervention (Ekici 2015). On the other hand, no changes in the seizure frequency were detected in a randomized control study including 37 patients with focal temporal lobe epilepsy, in whom the anode was placed on the projection of the dorsolateral prefrontal cortex in order to reduce depression-related symptoms. Moreover, none of the patients showed an increase in the frequency of seizures during the four-week follow-up period after the intervention (Liu et al. 2016).

In a review of 172 studies using tDCS, 56% of them reported adverse effects in both groups of patients: those who received stimulation and those who had placebo stimulation. In this framework, the adverse effects were tingling, sensation of burning, redness, and headache in all cases of low intensity and short duration (Brunoni et al. 2012).

Similarly, in a study performed by Fregni et al. (2006c) to examine the effects of cathode tDCS (one session, 20 min, 1 mA) in ten patients with drug-resistant epilepsy and cortical dysplasia, no adverse effects were observed (Fregni et al. 2006c). Further to this, in an investigation carried out by Auvichayapat et al. (2013) in pediatric patients with DRE, one of the 27 children who received active cathodic tDCS experienced erythematous rash below the reference electrode, that disappeared within 2 h after stimulation (Auvichayapat et al. 2013).

With regard to rTMS, the existence of seizures has been more frequently reported. After the establishment of the safety guide for the use of TMS in 1998 and until 2008, the occurrence of seizures as a consequence of this type of NIBS was indicated in nine reports. Four of them apparently occurred in patients in whom safe stimulation parameters were used, and three of them in patients who received pro-convulsant drugs. The remaining seizures happened under the use of stimulation protocols that were not considered to be safe (Liu et al. 2016). As a whole, the presence of seizures as a result of rTMS has been described in few patients with different neurological pathologies, most of them using high-frequency protocols (Bermppohl et al. 2006).

In 2007, Bae et al. analyzed 280 people with epilepsy in order to evaluate the safety and tolerance to rTMS. In this study, in a total of 152 epileptic patients who received rTMS ≤ 1 Hz sessions, no epileptic seizures related to stimulation were observed. The most frequent adverse effect was headache, occurring in 9.6% of the patients. On the other hand, epileptic seizure occurrence was confirmed in four patients. In 3 of them, typical seizures, considering duration and semiology, were reported. So, it is likely that the seizures did not have a causal relationship with the stimulation. Conversely, in the other patient, atypical seizures from the stimulation region during high-frequency rTMS were recognized, suggesting a causal connection between the stimulation and the occurrence of seizures. No rTMS-related episodes of status epilepticus were described in this review. All together, the authors in this study concluded that the risk of developing seizures was about 1.4% (Bae et al. 2007). Interestingly, findings generated in a recent review suggest that if seizures occurred, they are usually self-limiting, and the risk of TMS-related seizures is $<1\%$ overall. The rate of TMS-related seizures is comparable to that of most psychotropic

medications. Thus, most treatment recommendations for TMS-related seizures are supportive in nature (Stultz et al. 2020).

In sum, after more than three decades using tDCS, and more than 20 years applying TMS in human as well as in experimental models, along with the safety guidelines established by the International Federation of Clinical Neurophysiology, there is no evidence for irreversible injury produced by conventional stimulating protocols used to apply NIBS (Giustiniani et al. 2022; Rossi et al. 2021). These facts promote the idea that both rTMS and tDCS can be considered safe NIBS.

25.4 Noninvasive Brain Stimulation as Therapeutic Procedure: Effects on Seizures and Interictal Epileptiform Discharges in Drug-Resistant Epilepsy

Traditionally, studying clinical outcomes in epilepsy patients includes seizure frequency assessment. Yet, one of the main techniques to quantitatively measure the benefit of NIBS in these patients is focusing on change in the count of interictal epileptiform discharges (IEDs) using electrophysiological recordings.

Most of the studies show that tDCS results in a decrease of IEDs frequency. However, with regard to seizure frequency (SF), the findings are varied (Gschwind and Seeck 2016; Kwon et al. 2018; San-Juan et al. 2015). In this sense, Fregni et al. (2006a, b, c) used a cathodic tDCS protocol (one session, 20 min, 1 mA) in 19 patients with DRE due to dysplasia, reporting a decrease of 64.3% in IED, along with 44% of seizure reduction (Fregni et al. 2006c). Relatedly, in a control study involving 36 children with DRE using the same stimulation protocol, Auvichayapat et al. (2013) described that patients receiving tDCS showed a decrease in discharges by 45.3% immediately after the intervention, and 57.6% at 48 h; however, there were no significant changes in the frequency of seizures when compared with the control group (Auvichayapat et al. 2013). Two other studies reported significant (>50%) decrease in seizure frequency in patients suffering from Rasmussen's encephalitis (San-Juan et al. 2011; Tekturk et al. 2016). Another clinical pediatric trial conducted by Auvichayapat et al. (2016) has shown reduction in SF of 55.9% in patients with epileptic spasms and Lennox Gastaut syndrome (LGS) compared to sham group 1 month after 5 consecutive days of 20 min tDCS at 2 mA (Auvichayapat et al. 2016).

Later, 28 patients suffering from mesial temporal lobe epilepsy with hippocampal sclerosis were enrolled in a randomized placebo-controlled, double-blinded clinical trial where they received one session of tDCS at 2 mA for 3 or 5 days (San-Juan et al. 2017). Two months after the cathodal tDCS session, they obtained a decrease of -43% in SF for the group with a three-day stimulation and a decrease of -55% in SF for the group receiving tDCS for 5 days compared to baseline. So that, the heterogeneity of epilepsy types among studies demonstrates the potential efficacy of cathodal tDCS for treating several etiologies of refractory focal epilepsy.

It is recognized that the effect of the TMS is mediated by variables in terms of the stimulation frequency and the intensity of the stimulus. In this regard, most of the studies carried out to date in patients with DRE have applied rTMS at low frequency, and used different protocols including more than two treatment sessions (Eröss et al. 2015; San-Juan et al. 2017; Schulze-Bonhage 2019). Similar to the studies in which cathodic tDCS has been used, not all the investigations related to rTMS have reported how the frequency of IED behaves after the stimulation sessions have been applied (Lefaucheur et al. 2014, 2017). Nevertheless, some authors provide evidence of the significant inhibitory effect of rTMS on IEDs without clinical changes in the seizure frequency (Cantello et al. 2007; Joo 2012). In a control study developed by Sun et al. (2012), TMS was used at low frequency (0.5 Hz) in 60 patients with DRE. In the group where active stimulation was applied, the frequency of seizures decreased from 8.9 ± 11.1 per week to 1.8 ± 3.7 , while in the control group, no changes were observed (Sun et al. 2012).

Several studies have demonstrated that LF-rTMS may reduce seizure frequency in patients with refractory epilepsy (Fregni et al. 2005; Joo 2012; Lefaucheur et al. 2014). Noticeably, the findings derived from controlled trials are mixed in relation to antiepileptic rTMS efficacy (Cantello et al. 2007; Fregni et al. 2006b; Kwon et al. 2018; Theodore and Fisher 2004), and the field would benefit from further carefully randomized-controlled trials.

The variations in the results described in the literature regarding IEDs frequency and SF may be due to the great heterogeneity that exists in terms of clinical characteristics and treatment lines with ASD of the samples and the corresponding parameters of the stimulation protocols. Previous research findings indicate that IEDs are not necessarily generated in the ictal onset zone, but can be generated in a wider area that is called irritative zone (Hilz 2022).

In an experimental study in animals using deep brain stimulation, Sobayo and Mogul (2016) found that the stimulation was more effective when the stimulation parameters corresponded to the seizure characteristics of each animal in terms of location and frequency of termination (Sobayo and Mogul 2016). That said, it is probable that the personalization of NIBS protocols is the way to increase the effectiveness of these techniques, taking into account that the physiopathological and clinical characteristics, the location, and extension of the epileptogenic zone (EZ) and treatment with ASM are very fluctuating among patients with DRE.

Interestingly, brain modeling and human studies highlight the influence of individual brain anatomy and physiology on the electric field distribution (Simula et al. 2022). Recent advancements in vivo electric field characterization may enable clinical researchers to derive better relationships between the electric field strength and the clinical results. Also, subject-specific electric field simulations could lead to improved electrode placement and more efficient treatments. Accordingly, processing methods result in personalized NIBS based on metrics like focality and field strength, which allow for correlation with clinical outcomes (Beumer et al. 2022).

Unquestionably, there is a lack of clinical studies investigating changes in intracranial epileptiform discharges during NIBS application, which could make clear the nature of TMS and DCS-related local and network dynamics in epilepsy.

25.5 Evaluation of Non-invasive Brain Stimulation Effects on Electroencephalogram Functional Connectivity

There are well-known effects on functional connectivity in both normal and clinical populations as demonstrated in functional magnetic resonance imaging (fMRI) and Electroencephalogram (EEG) studies. Functional imaging techniques such as positron emission tomography, fMRI, and EEG mapping enable assessment of TMS-related functional brain activation (Pascual-Leone et al. 2011; Ruffini et al. 2014; Shafi et al. 2016). Studies combining resting-state functional magnetic resonance imaging with NIBS allow delineating how stimulation of different brain regions induces complex network modifications, both at the local and distal level. More recently, some studies involving magnetic resonance spectroscopy and NIBS have demonstrated how microscale changes are related to modifications of large-scale networks (Pini et al. 2018).

Altogether, a combination of TMS and functional imaging can be useful in three principal ways: (1) brain imaging before TMS is helpful in defining the accurate coil position over a distinct cortical area targeted by TMS; (2) imaging the brain during TMS is a promising approach for assessing cortical excitability and intracerebral functional connectivity; and (3) brain imaging after TMS can be employed to study the plasticity of the human cortex by evaluating lasting effects of TMS. Undoubtedly, this approach will help to advance our understanding of the therapeutical effects related to TMS (Siebner et al. 2009). Likewise, TMS has been also used in conjunction with EEG (TMS-EEG) to evaluate neurophysiology for a variety of indications. TMS-EEG has significant potential for exploring brain connectivity using focal TMS-evoked potentials and oscillations, which may allow for the system-specific delineation of neural recovery patterns after neurological diseases (Keser et al. 2022).

In relation with tDCS, Stagg et al. (2010) assessed the modulation of cerebral perfusion during and after tDCS application to the left dorsolateral prefrontal cortex. These authors described an increase in the perfusion of the primary sensory-motor cortex, cingulate cortex, and left parietal cortex as compared with baseline perfusion during stimulation with anodal tDCS. On the other hand, cathodal tDCS decreased perfusion in thalamus and in temporal lobe compared with baseline state (Stagg et al. 2010). Recent articles have reported changes of functional connectivity in epileptic patients after tDCS. Further to that, there is evidence that tDCS may act by affecting brain networks, rather than simply modifying local activity in the targeted area (Simula et al. 2022).

Nowadays, the analysis of functional connectivity and the application of graph theory to evaluate the behavior of neuronal networks in patients with DRE who have undergone some neuromodulation techniques constitute a new variant for the approach of the predictive value of the therapeutic response of the intervention, as well as the evaluation of its effect. Moreover, graph theory research has been progressively used to analyze brain networks in different structural and functional modalities (Chiang and Yang 2019; Pedersen et al. 2019). It is important to

highpoint that functional and structural connectivity clarifies not only that but also the extent to which different brain zones are connected, whereas network analysis using graph theory provides a framework to characterize the topological organization of functional and structural networks, before, during, and after the stimulation.

The most common parameters utilized in neuronal network analyses using graph theory are the clustering coefficient and the characteristic path length. The clustering coefficient allows to define the local segregation property of the network, and it is used to assess the network capability to share specialized data, while the path length and global efficiency are used to evaluate the capacity of the network as a whole for inner-exchange information. A short path length, a low clustering, and a high global efficiency/local efficiency generally represent a small world topology of the network and characterizes an optimal organization for communication efficiency (Morales Chacón et al. 2021a).

In a control study in patients with extratemporal focal epilepsy using functional magnetic resonance imaging (fMRI), Pedersen et al. (2015) observed increased segregation (clustering coefficient and local efficiency) compared to healthy subjects (Pedersen et al. 2015). In another investigation carried out by Antony et al. (2013), where an analysis of the functional connectivity derived from EEG was made to predict the post-surgical clinical evolution in patients with drug-resistant temporal lobe epilepsy, an increase in the clustering index and the average length was found precisely for the frequency bands slow (Pedersen et al. 2015).

Also, Tecchio et al. (2018) assessed the cathodic tDCS-induced changes of electroencephalography-derived brain functional connectivity using low-resolution electromagnetic tomography (eLORETA) in patients with temporal lobe epilepsy. The findings indicated that about 73% of the changes in functional connectivity involved the epileptogenic zone and that the reduction of seizures were correlated with the increase in functional connectivity (Tecchio et al. 2018). This study also supports the hypothesis that functional connectivity changes may contribute to explain the effects of tDCS in epilepsy, offering a new scenario in the personalization of neuromodulation interventions in epileptic people.

All things considered, research in patients with DRE using fMRI, EEG, magnetoencephalography, and electrocorticography has shown an increase in connectivity patterns around the epileptic zone. On the other hand, the connectivity of the epileptic zone with distant neural networks is diminished, and this is related to the duration and severity of the disease (Morales Chacón et al. 2021a). However, it is not entirely clear how these characteristics of neural networks could be modulated by NIBS.

25.6 Conclusions

As has been demonstrated in this chapter, NIBS can be considered a potential therapeutic alternative for patients with drug-resistant epilepsy who are *not* eligible for epilepsy surgery. However, NIBS antiepileptic efficacy will have to be determined in future randomized placebo-controlled trials. Equally, further multicenter clinical

studies will need to be undertaken to elucidate the effects of these strategies when combined with ASMs, measures of outcome assessment, and eligibility criteria of patients, including novel methods such as personalized stimulation protocols based on computational modeling.

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Chapter 26

Effects of Transcranial Focal Electrical Stimulation Via Concentric Ring Electrodes on Seizure Activity



Walter G. Besio

Abstract Epilepsy affects approximately one percent of the planet's population. There does not appear to be any single therapy that works for all types of epilepsies. As an alternative, we have been developing a noninvasive, or minimally invasive, transcranial focal electrical stimulation (TFS) based on the novel tripolar concentric ring electrode (TCRE). By applying biphasic, charge balanced, constant current, pulses noninvasively through the TCRE, we have realized acute seizure attenuation in rats. In different rat seizure models (penicillin, pilocarpine, pentylenetetrazol, 3-mercaptopropionic acid) and through hundreds of experiments, we have demonstrated that TFS successfully aborted seizures, and even *status epilepticus*, a severe form of seizure activity that is estimated to cause the death of 20,000 to 40,000 people each year in the US alone. Additionally, TFS selectively increased gamma-aminobutyric acid (GABA) and decreased glutamate extracellular levels concurrently. TFS also prevented naïve brains from epileptogenesis in the electrical amygdala kindling in cats, a larger animal model. TFS reduces the over-release of glutamate, brain damage, and Pgp overexpression and function induced by seizures. TFS augments the effects of phenytoin (PHT) in animals with PHT-resistant seizures. Further, TFS potentiated the effectiveness of diazepam. Lastly, TFS does not induce brain damage nor alter memory in rats or humans and does not cause sensation or pain, allowing truly double-blinded studies. In conclusion, we have found TFS to be effective at preventing, aborting, or attenuating acute seizures induced by penicillin, pilocarpine, pentylenetetrazol, and 3-mercaptopropionic acid and was safe. Lastly, TFS reverted the drug-resistant seizures (DRS) in rats, and we hope that it will have similar effects in humans. In the future, we need to test if TFS is tolerable, safe, and effective in humans with pharmacoresistant epilepsy.

Keywords Transcranial focal stimulation (TFS) · Tripolar concentric ring electrode (TCRE) · Noninvasive · Seizure · Penicillin · Pilocarpine · Pentylenetetrazole (PTZ) · Neuromodulation

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26.1 Introduction

26.1.1 *Medically Intractable Epilepsy and Its Consequences*

Presently, antiseizure medication is the primary treatment for epilepsy. However, approximately half of new-onset seizure patients are not cured by medical therapy (Kwan and Brodie 2000). An estimated 425,000 Americans suffer from pharmacoresistant epilepsy that does not respond to antiseizure medication (Kwan and Brodie 2000). Up to 25–30% of people with epilepsy will continue to have seizures despite optimal medical therapy (Sheng 2018). Uncontrolled seizures have been related to increased morbidity and mortality, resulting in an increased incidence of progressive developmental delay and sudden accidental deaths (Krumholz 1995). Approximately 1% of the world population (~67 million) suffers from epilepsy, of which 85% live in developing countries where medication may be too costly (World Health Organization). Each year in the United States, approximately 50,000 to 150,000 status epilepticus (SE) cases occur (Sirven and Waterhouse 2003) causing 22,000–42,000 deaths (Hocker 2015), with the mortality rate up to 38% (DeLorenzo 1996; Shorvon et al. 2007), and when persisting beyond one hour mortality has been reported at 65% (Drislane 2009). Many more deaths occur in developing countries.

26.1.2 *Brain Stimulation for Pharmacoresistant Epilepsy*

Brain stimulation is a promising technology for treating medically intractable epilepsy (Theodore and Fisher 2007). Electrical stimulation of the brain and its periphery has a long history (for reviews see (Thoma and Young 1993; George 2000a, b)). Over the past 30 years, applications have included cerebellar stimulation (Davis 2000), the vagus nerve stimulation (VNS) (George 2000a, b), and deep brain stimulation (DBS) targeting sites such as the subthalamic nucleus (Chabardes 2002), mesial temporal structures (Vonck 2002), and anterior thalamic nucleus.

Invasive Approaches

The VNS by Cyberonics, now LivaNova, was the only FDA-approved stimulation therapy for intractable epilepsy when the first edition was published. Several controlled trials have assessed the efficacy of VNS (VNS Study Group 1995) (Handforth 1995; Ben-Menchem 1994). Generally, it is found to be as effective as antiseizure medications for select seizure populations, and serious complications are uncommon (Ben-Menchem 1994).

Deep brain stimulation (DBS) by Medtronic is approved by the FDA to deliver electrical stimulation to structures in the brain that control movement and muscle function for movement disorders. Preliminary animal and clinical studies on DBS

have shown promise for seizure control. One study showed that DBS suppressed the secondary generalization of limbic seizures in rats (Usui 2005). There is also preliminary evidence that DBS led to clinical improvement in seizure control of refractory epilepsy patients (Velasco 2000; Vonck 2002; Kerrigan 2004). For invasive approaches complications such as hemorrhage are few, on average in about 5% of the patients (Fisher 2003; Bhatia 2011), and infection rates are similar with a 4.7% average (Ben-Haim 2009; Pourtian 2011). Dr. Fisher ran the controlled clinical trial of stimulation of the anterior nuclei of thalamus for epilepsy (SANTE) study (Fisher 2010). At the end of a 3-month blinded phase the stimulated group had a 29% greater reduction in seizures compared with the control group. After two years, there was a further reduction. At the end of 7 years, the median seizure frequency percent reduction from baseline was 75% (Salanova 2021). In 2018, a few years after the completion of SANTE trial, and the 7-year follow-up, DBS for epilepsy was approved by the FDA.

The responsive neurostimulator (RNS) by Neuropace delivers a short train of electrical pulses to the brain through implanted leads in response to detected abnormal electrical signals of the brain. Preliminary results on RNS have been encouraging. In a study on rats, RNS using low-frequency stimulation was shown to decrease the incidence of kindled seizures (Goodman et al. 2005). Analysis of individual cases suggested that RNS may have suppressed seizures in some patients (Kossoff 2004). Results from the RNS pivotal clinical trial suggest that there was a decrease in seizure frequency (Gigante and Goodman 2011). At the end of the two-year randomized multicenter double-blinded controlled trial of RNS, the median percent reduction in seizures was 53% (Heck 2014). VNS, DBS, and RNS all require expensive, invasive surgeries for implantation of electronics. Though the associated complication rates are comparable to the norm in neurosurgical practice, they do represent increased risks.

Noninvasive Approaches

Efforts to interrupt epileptic seizures noninvasively include transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS), and electroconvulsive therapy (ECT).

TMS applies magnetic fields to the cranium, producing electric fields in the brain. TMS has been studied extensively over the past decade with mixed results for controlling seizures and remains an experimental device (Hallett 2002; Theodore 2002; Tassinari 2003). TMS in one study showed effectiveness for malformations where the seizure focus could be localized in the neocortex (Fregni 2006a, b). TMS can reach into the cortex up to 2–3 cm below the scalp (George 2003). In the future, coils may be developed that stimulate at greater depth; however, to increase depth, they will have less focus (Zangen 2005).

Transcranial electrical stimulation (TES) was used safely back in 1980 to noninvasively stimulate the cortex (Merton and Morton 1980). Limited pilot studies showed that two forms of TES – tDCS and ECT – might have antiseizure effects on

selected patients. Unfortunately, for either case, the stimulation is difficult to focus. In a pilot study on patients with malformations of cortical development and refractory epilepsy, tDCS demonstrated a decrease of epileptic discharges but no significant reduction in the number of seizures (Fregni 2006a, b). For tDCS to be effective, the cathodic electrode is placed over an identified seizure focus and the anodic electrode placed far away from it. According to a systematic review of tDCS results, tDCS is safe and probably effective for epilepsy (Sudbrack-Oliveira 2021). However, there need to be larger sham-controlled randomized trials to verify efficacy (Sudbrack-Oliveira 2021). Although tDCS is safe (Fregni 2006a, b) the charge passage is not balanced.

Electroconvulsive therapy is typically used for severe depression (Chanpattana and Sackeim 2010). It induces a controlled seizure by applying electrical pulses to an anesthetized patient's head. A case study reported that ECT acutely controlled seizures in two children (Griesemer 1997). Present-day ECT has limited spatial distribution of electrical current or dosage in the brain. Sackeim (Sackeim 2004) proposed a method to improve the focus of ECT. DeGiorgio also assessed the efficacy of stimulating the trigeminal nerve noninvasively in humans (DeGiorgio 2003). To summarize, brain stimulation for epilepsy has shown promise but requires further research. The best structures to stimulate and the most effective stimuli to use are still unknown (Theodore and Fisher 2007).

26.2 TCRES and TFS

We have developed a unique, noninvasive, or minimally invasive, method of stimulation: transcranial focal stimulation (TFS) via novel TCRES. TFS delivers focal stimulation, providing the opportunity to target specific brain regions at depths of a few centimeters (Khatoun et al. 2019). Reciprocally, the TCRES also allow high-fidelity focal recordings of neural activity. We have demonstrated that TFS via unique TCRES (transcutaneously and transcranially) successfully abolished or reduced experimentally-induced acute seizures and SE in rats, with minimal or no side effects (Besio et al. 2007, 2008, 2010a, b; Besio 2009, 2011a, b, 2013a, b; Makeyev 2012, 2013; Perez-Perez 2019). For example, TFS via TCRES abolished pilocarpine-induced SE seizures and prevented them from returning even hours after the stimulation was stopped without using any antiseizure medications such as diazepam (Besio et al. 2007).

26.2.1 Innovation

The innovative nature of TFS via TCRES hinges on three core distinctions from existing technologies and methods: (a) An innovative electrode design (TCRE), (b) providing noninvasive focal stimulation modality that has been demonstrated to

Fig. 26.1 Conventional disc electrode (left) and tripolar concentric ring electrode (TCRE) (right)



have antiseizure effects in acute seizure models, and (c) allowing for both focal stimulation and recording from the same electrodes.

Innovative Electrode Design

The invention of the novel TCRE is a key to this new noninvasive stimulation modality. We say noninvasive, but the electrodes can be used in minimally invasive and invasive applications as well. The TCRE consists of three electrode elements—outer ring, middle ring, and the center disc (Fig. 26.1). The invention of the TCRE is a significant improvement over the conventional disc electrode. Its details have been vetted in the peer-reviewed scientific literature (Besio and Fasiuddin 2005; Besio 2006; Koka and Besio 2007; Besio et al. 2007, 2008). TCREs have unique capabilities as they perform the second spatial derivative, i.e. the Laplacian, on the surface potentials (Fig. 26.1).

Focal Stimulation

When concentric electrodes are used for stimulation, by reciprocity, the current density achieved by driving current through these electrodes yields a similar focused stimulation in the tissue below (Wiley and Webster 1982a, b; Van Oosterom and Strackee 1983). With a particular balance of current among the three electrodes in the TCRE, we achieve focused stimulation into the tissue even through the skull, which is advantageous over the diffuse stimulation achieved by disc electrical stimulation applied across the head (Wiley and Webster 1982a, b; Van Oosterom and Strackee 1983).

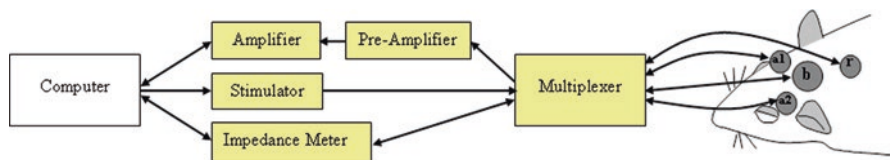


Fig. 26.2 Schematic of animal experimental set up. A laptop computer controlled the data acquisition. Electrodes (TCREs) on the rat were used for recording and stimulation. The multiplexer determined the direction of the flow, from the rat or to the rat. For recording, the signals flowed from the rat through the multiplexer to the preamplifier, amplifier, and then computer. The transcranial focal stimulation (TFS) flowed the other way from the computer to the stimulator and through the multiplexer to the TCRE and rat

Common Instrumentation for Focal Stimulation and Focal Transcranial Recordings from the Same Electrodes

We have developed custom instrumentation that can deliver TFS, and record Laplacian EEG and conventional EEG, all from the same TCRE. A block diagram of our custom-designed TFS instrumentation system is shown in Fig. 26.2. The TCREs were placed on the scalp with conductive paste. One TCRE (b) (diameter = 10 mm), used to record from and stimulate, was centered on the top of the rat head, behind the eyes. Two other recording electrodes (a1, a2) (diameter = 6 mm) were placed bilaterally behind the eyes, closer to the subcortical structures such as the hippocampus. A reference electrode (r) was attached to the top of the neck behind the ears. The electrodes were fixed in place using dental cement (Fig. 26.2).

26.3 Results from Animal Models

TFS successfully abolished seizures or reduced seizure severity in four different acute seizures models (Besio et al. 2007, 2008, 2010a, b; Besio 2009, 2011a, b, 2013a, b; Makeyev 2012, 2013; Perez-Perez 2019, 2021; Santana-Gómez 2015a, b).

26.3.1 Penicillin

When TFS was triggered manually after severe penicillin-induced myoclonic jerks, there was a significant reduction in the number and length of myoclonic jerks (Besio 2009; Besio et al. 2010a, b). A small amount (approximately 0.2 cc) of cerebrospinal fluid was removed and replaced with penicillin G (2.5 MU/kg) (Besio 2009; Besio et al. 2010a, b). Within 1 min of the penicillin injection, on average, myoclonic jerks began. Once the myoclonic jerks reached the rate of 30/min, TFS was applied to the electrode (b) Fig. 26.2. In the control group ($n = 8$), not receiving TFS, the average

maximal myoclonic jerk rate was 70/min with an average duration of 90 minutes. In the experimental group ($n = 17$), various pulse widths and frequencies were examined. There was a significant decrease in the mean myoclonic jerk rate from 41/minute to 21/minute ($P < 0.0001$, two-sample t-test) after the first application of TFS. After TFS was applied, myoclonic jerks stopped in all instances for a few minutes and then returned with a smaller amplitude and a lower frequency. In 13 cases, repeated stimulation led to a complete cessation of myoclonic jerks. These results gave us hope that TFS had antiseizure effects. However, this seizure model is not a commonly used model, and so we began work with the pilocarpine SE model.

26.3.2 *Pilocarpine*

To test the antiseizure effects of TFS in a more common model, we decided to use pilocarpine, a muscarinic receptor agonist model of <http://en.wikipedia.org/wiki/Pilocarpine> SE. TFS via TCRES led to a significant reduction in the intensity of pilocarpine-induced SE (an extreme form of seizures that is estimated to take 22,000 to 40,000 lives in the United States. annually), with the effects lasting hours (Besio et al. 2007). Thirty minutes after the administration of scopolamine methylnitrate, pilocarpine HCl (310 mg/kg, i.p., Sigma) was administered. Rats were randomly assigned to one of two groups: control ($n = 8$) and experimental ($n = 8$). Symmetrical, biphasic, charge-balanced, constant current TFS pulses were applied to experimental rats via our custom-made stimulator. TFS was delivered via the outer ring and disc (with the middle ring floating) of the electrode at location (b) shown in Fig. 26.2. The range of TFS parameters used was 200, 300, 500, or 750 Hz, 200 or 300 μ s pulse duration, and 50 or 60 mA intensity, applied for 1 min. TFS was started with the least intense parameter set (200 Hz, 200 μ s pulse width, 50 mA) and progressively increased if there was no obvious change in electrographic and/or behavioral activity. Control rats did not receive TFS. Example traces from this study are shown in Fig. 26.3. Note that, without additional pharmacological intervention, the electrographic and behavioral activity did not return for hours. There was a significant improvement in survival for the TFS-treated animals compared to those without the application of TFS due to the pilocarpine-induced status epilepticus. Long-lasting control of SE, without antiseizure medications, provided positive proof that TFS had antiseizure effects.

Effects of Transcranial Focal Electrical Stimulation Alone and Associated with a Subeffective Dose of Diazepam on Pilocarpine-Induced Status Epilepticus and Subsequent Neuronal Damage in Rats

The use of brain stimulation in the treatment of pharmacoresistant epilepsy has a long history, but few studies have focused on its acute effects to prevent status epilepticus (SE) and its consequences. The identification of therapeutic strategies

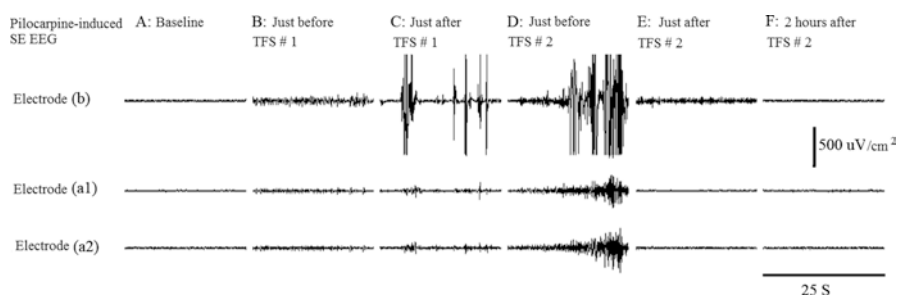


Fig. 26.3 Three traces of electroencephalography (EEG) from a rat in the transcranial focal stimulation (TFS) group. (a) Baseline recordings before pilocarpine introduction. (b) Five-minutes after status epilepticus (SE) started. (c) Just after 2 minutes of TFS was applied. (d) five-minutes after the first dose of TFS, the SE electrographic activity is high. (e) Immediately after the second TFS does the electrographic activity, and behavioral activity, were reduced toward baseline. (f) Two-hours after the second dose of TFS the activity is still like baseline, even though the pilocarpine is still very active in control animals at this time of the experiments

that prevent SE and its consequences constitutes a major clinical need. Therefore, for the present study, we investigated if TFS associated with diazepam (DZP) may represent a good approach to avoid the expression of this disorder and the subsequent neuronal damage. Experiments in rats were designed to investigate if TFS alone or associated with a subeffective dose of DZP was able to prevent the lithium-pilocarpine-induced (LP) SE and consequent cell damage in the hippocampus when applied before the pilocarpine injection.

We included six groups of rats in this study (Besio 2013a, b). The groups were: (1) LP-TFS-DZP, this group is the experimental group to test if TFS combined with a subeffective dose of DZP can significantly alter the effects of the LP; (2) LP-DZP, this group is a control group for the LP-TFS-DZP group; (3) LP-TFS, this group is to determine if TFS alone has significant effects on LP-induced seizures; (4) LP, this group controls groups 1–3; (5) TFS, to verify what affects TFS and SS have, and lastly, (6) A control group that only receives SS.

The animals from groups 5 (TFS) and 6 (Control) did not show any behavioral changes after the manipulations. Histology was performed using the floral jade technique and showed very few degenerating neurons in the hippocampus for either group. Nearly all the animals from the LP group (95.8%) showed mild and severe generalized seizures, which evolved into SE. In all rats that went into SE, we observed extensive FJ+ staining in CA1, CA3, and hilus of dentate gyrus. A lower percentage of animals from the LP-TFS group showed mild (78.5%) and severe generalized seizures (78.5%) as well as SE (71.4%). However, these values as well as latencies to the first forelimb clonus, first generalized seizure and establishment of SE were not significantly different when compared with the LP group. The LP-TFS group demonstrated a lower number of FJ+ neurons, an effect that was significant in CA3 (20%, $p < 0.01$) and dentate gyrus (16%, $p < 0.05$) of rats without SE, when compared with the LP group. All rats pretreated with a subeffective dose of DZP (LP-DZP group) had LP-induced seizures and SE, and nonsignificant

changes were found in latencies to the different behavioral alterations when compared to the LP group. In contrast with the LP group, rats from the LP-DZP group demonstrated a significant reduction in the number of FJ+ neurons in all the hippocampal areas evaluated. The pretreatment with a subeffective dose of DZP combined with TFS produced total protection against LP-induced seizures and SE in 61.6% of animals of the LP-TFS + DZP group, an effect that was significant when compared with the LP and LP-DZP groups and nearly significant for protecting against mild and generalized seizures in contrast with the LP-TFS group. Animals from the LP-TFS-DZP group that went into SE also demonstrated significantly increased latencies to the first forelimb clonus ($p < 0.001$), generalized seizure ($p < 0.001$), and establishment of SE ($p < 0.001$), when compared with the LP, LP-DZP and LP-TFS groups. Histological evaluation revealed a significant diminution in the number of FJ+ neurons in all hippocampal areas examined of animals from the LP-TFS-DZP group, a situation that was more evident when values were compared with the LP and LP-DZP groups. Analysis revealed that rats from the LP-TFS-DZP group had total protection against LP-induced seizures and exhibited a similar number of degenerating cells as the control group.

The results of the present study reveal that TFS applied before pilocarpine administration, by itself, induces a trend toward its effectiveness for preventing SE and neuronal damage. The effects are statistically significant when TFS neuromodulation is combined with subeffective doses of DZP. Our data support the notion that TFS combined with DZP can represent a good noninvasive prophylactic strategy to avoid or reduce the expression of seizure activity and neuronal damage induced by SE. Our results indicate that TFS can potentiate the antiseizure and neuroprotective effects mediated by the pretreatment with subeffective doses of DZP. This situation may be explained by an augmented GABA-gated chloride influx through GABA_A receptor-regulated ion channels that restricts interictal spike propagation under epileptic conditions and maintains an inhibitory input sufficient for neuronal survival. An important finding from this study was that TFS alone was able to reduce the LP-induced neuronal damage in CA3 and dentate gyrus, but not in CA1, of those animals without SE. In contrast, all rats receiving TFS plus DZP showed a significant reduction in the neuronal damage of CA1, CA3, and dentate gyrus subsequent to pilocarpine administration, even though some of the rats went into SE. These results support the idea that subeffective doses of DZP plus TFS can represent a good strategy to prevent SE and neuronal damage subsequent to brain insults.

Transcranial Focal Electrical Stimulation Reduces the Convulsive Expression and Amino Acid Release in the Hippocampus During Pilocarpine-Induced Status Epilepticus in Rats

In this study, we used microdialysis to quantify the effects of TFS on extracellular neurotransmitter concentrations in the hippocampus. Rats were implanted with a microdialysis cannula and a depth electrode in the hippocampus, and a tripolar

electrode on the skull for TFS (Santana-Gómez 2015a, b). Screws used for anchoring the dental acrylic were used for reference and recording. After a week of recovery, increasing iterative currents were applied to the tripolar electrode, with after discharges monitored from the recording electrodes. When the current caused after discharges were found, a current of 20% less was used for the microdialysis experiments. The subthreshold TFS was applied for 30 minutes with microdialysis performed for two-hours prior and post to the TFS. Another group that had microdialysis cannulas also received LP and TFS to evaluate extracellular hippocampus neurotransmitter concentrations from SE and how TFS affected the neurotransmitters.

Histological analysis showed that the depth electrode tips were located in the ventral hippocampus. During the control and basal conditions, the extracellular levels of GABA and glutamate were stable. After discharge, thresholds prior and postexperiment were not significantly different. There was a significant correlation between the current intensity of TFS and the changes in GABA and glutamate release. At current intensities of 2800 μ A or lower for TFS, decreased extracellular levels of glutamate and increased GABA were detected, with effects being more evident at the lower intensities (glutamate, 76% decrease at 400 μ A; GABA, 90% increase at 620 μ A). Administration of pilocarpine caused progressive behavioral changes that culminated in SE that occurred at 34.3 ± 5.5 min in 100% of the animals. After the initiation of the SE, animals from the SE group demonstrated a significant progressive increase in GABA and glutamate release and reached 120% ($p < 0.001$) and 182% ($p < 0.001$), respectively, by the end of the microdialysis experiment (240 min after the beginning of the SE). When compared with the SE group, the rats receiving TFS (SE-TFS group) demonstrated the lower intensity of the behavioral seizure activity, and none experienced wet dog shakes during the SE. Further, the amino acid release, SE-TFS group, had a nonsignificant increase of glutamate (44%, $p = 0.182$) and decrease in GABA (24%, $p = 0.154$) at the onset of the SE. Thereafter, no significant changes were detected during (glutamate, 5%, $p = 0.911$; GABA, 21%, $p = 0.171$) and 1 h after the end of the TFS (glutamate, 24%, $p = 0.700$; 15%, $p = 0.716$).

The present study indicates that TFS applied during SE reduces seizure activity, an effect associated with decreased power in 4- to 8-Hz (theta) and 30- to 90-Hz (gamma) bands in the hippocampus. These results correlated with a significant decrease of SE-evoked increases in GABA and glutamate release. This study revealed that TFS applied at low current intensities results in enhanced GABA and decreased glutamate extracellular levels in the hippocampus. According to these changes, it is expected that TFS protects the animals from the seizure activity when applied before a proconvulsant stimulus in normal animals. We found that TFS avoided increases in the release of glutamate during the SE, an effect that can explain the reduced seizure activity of animals receiving TFS after the initiation of the ictal event induced by pilocarpine, pentylenetetrazol, and penicillin G (Besio et al. 2007, 2010a, b). Our microdialysis experiments revealed that the extracellular levels of GABA remained without changes when TFS was applied during SE. Although it is possible to assume that this circumstance could contribute to an

intensification of the SE, we found reduced seizure activity. This evidence suggests that the antiseizure effects induced by TFS depend on the rate of brain excitability. A similar condition is also observed when phenytoin blocks the development of seizure activity in subjects with high-frequency cerebral firing, while it does not have this effect in subjects with normal brain activity associated with low neuronal firing rates (Yaari et al. 1986).

26.3.3 *Pentylentetrazol*

TFS Reduced PTZ-Induced Hypersynchrony

TFS via TCRES significantly reduced PTZ-induced hypersynchrony at the beta and gamma frequencies (Besio 2011a, b), as quantified from cross-channel coherence (CCC), which is similar to correlation; however, it is performed in the frequency domain rather than the time domain. Rat behavior was scored for seizure-related phenomena (Mirski 1997), and TFS was administered immediately after the first myoclonic jerk was observed. In each of the three electrode combinations the Pre-TFS CCC, which was calculated from the signals recorded after administering the PTZ and just prior to applying the TFS, was consistently high over the full frequency range tested 1–50 Hz. The baseline (prior to administration of PTZ) and Post-TFS (just after the TFS was terminated) CCC values were similar and lower than during the Pre-TFS stage. As shown in Fig. 26.4, for electrodes (a1) and (b), the CCC values after the application of TFS (blue/solid) return to pre-PTZ measured baseline values (green/dashed), in contrast to those prior to treatment (red/dotted). This was

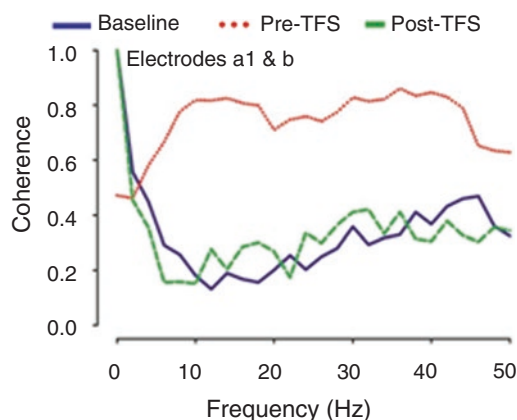


Fig. 26.4 The three traces show the coherence, correlation in the frequency domain, between the EEG. The solid line is baseline with very little coherence above 10 Hz. The dotted line shows after PTZ was introduced to the animal and the seizure caused synchronization of the EEG the coherence is high. The dashed line shows immediately after applying the TFS the coherence, a sign of brain synchronization, is reverted back towards baseline

indicative for all electrode combinations and all rats. The high coherence suggests that there was a synchronization of brain electrographic activity over a wide area of the brain during the seizure. In contrast, after the application of TFS, the CCC was reduced, suggesting that the synchronized activity was reduced by TFS.

For recording signals, we have previously shown that TCREs, compared to conventional disc electrodes, provide less than one-tenth (8.27%) of the mutual information (Koka and Besio 2007) and have strong attenuation of distant sources, -100 dB one radius from the electrode (Besio 2006). The reduced mutual information and strong attenuation of distant sources infer that during the PTZ-induced seizures, since there was a high CCC between all electrodes, there is highly synchronized activity between major areas of the brain (Fig. 26.4).

TSF Reduced Two-Dose PTZ-Induced Behavioral Activity

In this study we expanded our analysis of the effect of TFS on behavioral seizure activity in two ways. First, four different metrics were used: (1) time of the first behavioral change, (2) seizure onset latency, (3) seizure duration, and (4) maximal seizure severity score. This allowed for a better description of the effect of TFS. Second, in our preliminary PTZ study, two independent groups of animals were used (Besio et al. 2010a, b). The PTZ was administered to animals in both groups only once, with only the TFS-treated group receiving TFS but not the control group. This previous approach did not account for variability (resistance to PTZ, etc) among the animals of the two groups, potentially obscuring the effect of TFS. To account for this variability, in this study, we used a different experimental design administering PTZ to the animals in both groups twice and giving TFS to the animals in the TFS-treated group after the second PTZ administration only. This approach allowed us to compare the results after the first PTZ administration in the TFS-treated and control groups, confirming that there was no significant difference between controls and TFS-treated groups. The results from the first application of PTZ and the resulting first seizure were considered the baseline to study the difference between the first and the second PTZ-induced seizures in each group separately. Finally, we compared the changes caused by recurrent PTZ administrations in control and TFS-treated groups to evaluate the effect of TFS.

The effect of recurrent administrations of PTZ producing a gradual increase in the seizure intensity is well established and used for the development of PTZ-induced kindling in rats (Ito et al. 1977; Corda, Biochem; Han 2000; Szyndler 2002). Recurrent intraperitoneal administration of doses equal to 30 mg/kg (Corda, Biochem), 35 mg/kg (Szyndler 2002), and 40 mg/kg (Han 2000), comparable to the dose used for our study (45 mg/kg), were shown to produce progressive sensitization to the convulsive effect of PTZ. Due to this sensitization effect of PTZ, it would be difficult to reliably evaluate the effect of TFS using the same animals first as a part of the TFS-treated group and then as a control or vice versa. Even though including the animals in both legs of the study, as controls and then again in the treated group or vice versa, allows for a within-subject comparison, it also convolutes the effect of

TFS with the increased sensitivity to PTZ. This sensitization along with the possible obscuring/masking of PTZ tolerance when using a single PTZ administration on two separate groups of animals, was why we administered two doses of PTZ in both groups. We compared the effect of TFS between the first and the second PTZ administrations in control and TFS-treated groups separately to overcome these limitations.

We found no significant differences in behavioral activity from the first PTZ treatment between the two groups (Makeyev 2013). Therefore, both groups had similar behavioral activity induced by the first administration of PTZ. It should also be noted that after the second administration of PTZ, the TFS would not have had any effect on the time of the first behavioral change since the TFS was not turned on until the first behavioral change was observed. Therefore, until the time of the first behavioral change, both groups were treated the same and were not found to be statistically different. We then compared the difference between the first and the second PTZ-induced seizures in each group separately. The same behavioral seizure activity metrics (seizure onset latency, time of the first behavioral change, duration of seizure, and maximal seizure severity score) were used in both cases. There was a significant difference in all four metrics between the first and second PTZ-induced seizures for the control group. While the general trend was the same in the TFS-treated group (mean/median decrease in time of the first behavioral change, decrease in seizure onset latency, increase in seizure duration, and increase in maximal seizure severity score), the difference was statistically significant only for the time of first behavioral change, that could not have been affected by TFS in the TFS-treated group (Makeyev 2013).

The fact that there was no statistically significant change in three behavioral seizure activity metrics that could have been affected by TFS clearly suggests that TFS may have an antiseizure effect. If this difference between the two groups was due to some factor other than TFS, all four behavioral seizure activity metrics affected or not affected by TFS would have been likely to exhibit similar behavior.

Automated Seizure Detection Triggers TSF and Reduces PTZ-Induced Electrographic Activity

After having previously shown that TFS was effective in altering convulsant-induced seizures we decided to determine if it was feasible to noninvasively and automatically control PTZ-induced seizures. We developed real-time seizure detectors using noninvasive electrographic seizure activity from TCRES based on a disjunctive combination of the cumulative sum (CUSUM) algorithm and generalized likelihood ratio test (GLRT). The seizure detectors automatically triggered TFS. The CUSUM is a signal change detector traditionally used in quality control, intrusion detection, spam filtering, and medical systems to identify changes in probability distribution of a stochastic random process. Although there is no optimality associated with the GLRT, it has been shown to work well in practice. We performed experiments following the methodology proposed by Makeyev (2011) and Besio

(2011a, b), Makeyev (2012) to confirm the effect of automatically triggered TFS on PTZ-induced electrographic seizure activity in rats. We applied the CUSUM and GLRT to verify the change in power between the two data segments.

The performance of the two detectors, CUSUM and GLRT, was comparable in terms of all the performance metrics. The GLRT performed slightly better, especially on sham seizure (specificity of 97.66% compared with 91.9% for CUSUM). The improved specificity is important since, in real-life applications, a false positive detection may mean an extra dose of electrical stimulation or a dose of antiseizure medication. As a result of a tradeoff, higher specificities mean lower sensitivities, however even with the sensitivity of 33.73% for a disjunctive combination (logical OR) of all three detectors, the seizure onset was detected prior to the first PTZ-induced myoclonic jerk in 76.92% of rats of the test set ($n = 13$). According to the GLRT analysis comparing segments of comparable time between the control rats and TFS-treated rats, the automatically triggered TFS significantly ($p = 0.001$) reduced the electrographic seizure activity power in the single dose TFS-treated group ($n = 5$) compared to controls ($n = 4$), in 70% of the paired segments further suggesting its antiseizure effect. It was also observed that the second automated dose of TFS reduced the electrographic activity even further toward the baseline.

Effects of Transcranial Focal Electrical Stimulation Via Tripolar Concentric Ring Electrodes on Pentylentetrazole-Induced Seizures in Rats

This study was conducted to evaluate the effects of noninvasive transcranial focal electrical stimulation (TFS) via tripolar concentric ring electrodes (TCRE) on the electrographic and behavioral activity from pentylentetrazole (PTZ)-induced seizures in rats (Besio 2013a, b). In this study, we used a longer electrographic activity time windows (15 min long) analyzed to confirm if TFS has long-lasting effects. For behavioral activity, four different metrics were used including the latency of the seizure onset, the number of myoclonic jerks (MJs), the duration of myoclonic activity, and the maximal behavioral seizure activity score to better assess the effect(s) of TFS on the PTZ-induced acute seizures.

The animals had the electrodes placed on their scalp the day before the experiment. On the day of the experiment, the impedance of the stimulation electrode was measured. If the skin-to-electrode impedance was greater than 10k Ω , the animals became controls, receiving i.p. PTZ and no TFS. If skin-to-electrode impedance was less than 10 k Ω the animals were in the TFS test groups with EEG or behavioral activity recording. Next, baseline EEG was collected for 15 minutes. After the baseline recording, the PTZ was given, and either behavioral activity or electrographic activity was recorded.

Electrographic seizure activity preceded behavioral activity and appeared as clear short high-frequency bursts and sometimes continued after the behavioral activity had ceased. After the administration of PTZ, the power spectral density in both the control and TFS-treated rats increased in rats that received TFS, the power

spectral density decreased immediately. However, the power spectral density in the controls stayed high. There was a significant decrease in power between the TFS-treated rats after receiving TFS compared to the control rats. For the behavioral activity, there were significantly less: myoclonic jerks and duration of behavioral activity in the TFS-treated group compared to the control group. The seizure onset latency and maximal seizure stage were not significantly different between the two groups. This study showed both electrographically and behaviorally that TFS had significant effects on PTZ-induced acute seizures in rats (Besio 2013a, b).

26.3.4 3-Mercaptopropionic Acid (MPA)

Noninvasive Transcranial Focal Stimulation Affects the Convulsive Seizure-Induced P-Glycoprotein Expression and Function in Rats

In drug-resistant epilepsy (DRE) there is an overexpression of inflammatory pathways (Weidner Lora 2018), enhanced oxidative stress (Pedre Lourdes 2018), and high release of glutamate (Luna-Munguia et al. 2011; During and Spencer 1993). These conditions induce P-glycoprotein (Pgp) overexpression (Bankstahl Jens 2008; Bauer 2008; van Vliet 2010; Deng 2018; Felix and Barrand 2002). Overexpression of Pgp is present in brain cell tissue from patients with DRE (Marchi 2004; Lazarowski 2007). Pgp overexpression in blood-brain barrier of patients with DRE limits the entrance from blood to cerebral parenchyma of some antiseizure medications (ASMs) (Tang et al. 2017; Zhang 2012). In addition, Pgp overexpression causes progressive seizure-related membrane depolarization (Auzmendi 2013), that facilitates the drug-resistant phenotype in epilepsy (Luna-Tortós 2008). The administration of Pgp inhibitors such as tariquidar or nimodipine reverses the drug-resistant phenotype (Brandt 2006; Höcht 2007, 2009; Van Vliet Erwin 2006). However, these drugs induce side effects and their clinical use is inconclusive (Asadi-Pooya Ali 2013; Borlot 2014; Elkhayat 2017; Narayanan 2016). We hypothesized that since TFS reduces extracellular glutamate (Santana-Gómez 2015a, b), there might be an effect on the Pgp.

The repetitive induction of generalized seizures with MPA results in Pgp overexpression and reduces brain bioavailability and antiseizure effects of phenytoin (PHT), producing drug-resistant seizures (Enrique 2017; de la Rosillo 2015). In experiment 1, we tested if TFS might be able to prevent the overexpression of Pgp and reduce seizure severity from a single acute seizure induced by MPA. We had four groups of male Wistar rats: (1) TFS-MPA that received both TFS and MPA, (2) MPA that only received MPA, (3) TFS that only received TFS, and (4) SS only received saline solution and were controls. Western blotting was performed to quantify the Pgp content. In experiment 2, we tested if TFS could revert the drug-resistant seizures. Once the rats had completed the repetitive induction stage, they were randomly admitted to one of four groups for these experiments: (1) PHT-TFS-MPA to test if TFS can inhibit the drug-resistant condition, (2) PHT-MPA this group

is to confirm that PHT did not induce antiseizure effects in animals with drug-resistant seizures, (3) TFS-MPA to test if TFS alone was able to modify the drug-resistant phenotype, and (4) MPA is the control for the other groups.

In experiment 1, the SS group rats did not show behavioral changes, as they should not have since they were controls. The Pgp expression in their cerebral cortex and hippocampus was used as a control condition for the other groups. All animals from the MPA group presented minor and major seizures after MPA administration. The MPA group showed significant overexpression of Pgp in both the cerebral cortex and the hippocampus. In the TFS-MPA group, the major seizures were reduced in some rats, but not enough for significance. However, the Pgp expression was similar to the SS group both in the cerebral cortex and hippocampus.

In experiment 2, all animals in the MPA group had minor and major seizures, with a maximum seizure stage of 4.3 ± 0.2 . The TFS-MPA group had less animals reaching major seizure stages but an insignificant number. The average seizure score was 4.0 ± 0.2 . In the PHT-MPA group, all animals had minor and major seizures. However, the latency to the major seizures was significant compared to MPA. For the PHT-TFS-MPA group, all animals had minor seizures, but major seizures in only 50% of the rats which was significant. Further, the average seizure score was 3.2 ± 0.04 , which was significant between the MPA and PHT-MPA groups.

TFS alone does not stop the seizures of MPA. Further, TFS alone did not revert the drug-resistant phenotype of the animals. However, TFS combined with PHT was able to reduce the expression of the MPA-induced major seizures. In conclusion, Pgp is overexpressed in the cerebral cortex and hippocampus of rats after an acute convulsive seizure, an effect avoided by TFS. In addition, the data obtained support that TFS facilitates the effects of PHT in an experimental model of drug-resistant seizures.

26.3.5 *Amygdala Kindling*

Transcranial Focal Electrical Stimulation via Concentric Ring Electrodes in Freely Moving Cats: Antiepileptogenic and Postictal Effects

Despite the previously reported benefits of TFS, all previous experiments have been performed on rats, and the effects of TFS on epileptogenesis are still unknown. In this study (Valdés-Cruz 2019), we used the electrical amygdaloid kindling (AK) model of temporal lobe epilepsy epileptogenesis, which consists of repeated and periodic electrical stimulations to limbic brain structures, progressively leading to the induction of an electroencephalographic after discharge (AD) and focal and secondarily generalized seizures (Sato et al. 1990). The kindling model is a chronic model that is currently used by antiepileptic drug discovery programs, including the NIH/NINDS-sponsored anticonvulsant drug development program in the U.S., and adequately predicts the clinical utility of novel antiseizure medications against

partial seizures in patients with epilepsy (Bialer and White 2010). This chronic model of epileptogenesis provides an opportunity to study the focal activation of a specific stimulated brain area and the gradual development of seizures in a larger animal model (Gorter et al. 2016). Thus, the aim of this study was to investigate the effect of TFS application via TCRES stimulating extracranially on convulsive activity and epileptogenesis induced by AK in cats (Valdés-Cruz 2019).

During surgery, stainless steel bipolar electrodes were stereotactically implanted (Snider and Niemer 1961), oriented to both amygdalae (AM) (AP +11.5, L 9.5, H 15.0). Epidural electrodes were implanted in both prefrontal cortices for electrographic recording. In addition, a TCRES (10 mm diameter) was fixed on the skull. The TCRES was fixed over the temporal bone (AP +11.5, L 5.5), ipsilateral to the kindled AM. In the control group, the TCRES were not placed, and the cats did not receive TFS. The cats were allowed to recover for 15 days in sound-isolated chambers used for recordings with food and water as libitum prior to the start of the experimental procedure. Thresholds were found for the AK stimulation and the TFS. The experiments were started on the following day of the threshold determination. AK stimulation every 24 h. TFS was administered for 2 min after the AK onset for 40 days, and then, only AK was applied. The control group only received AK stimulation. This procedure continued until all animals exhibited three consecutive kindling stage VI seizures, according to the Wada and Sato classification scheme (Wada and Sato 1974). Video and electrographic activities were recorded for later review by blinded observers.

The threshold intensity used for AK was 300–500 μ A and was kept constant until the animals were fully kindled. For tripolar TFS, the threshold intensity was 2.5 mA in all TFS-treated animals. Tripolar TFS after AK-treated animals reached stage VI after 80.0 ± 15.42 AK stimulations. Tripolar TFS after AK application significantly retarded the fully kindled state compared to the progression in control animals 25.4 ± 2.7 ($P < 0.001$). A significant increase in kindling stage II was observed in tripolar TFS after AK animals (38.4 ± 19.62) compared to that seen in controls ($P < 0.001$). For the epileptogenesis analysis, tripolar TFS after AK application significantly delayed the progression of behavioral seizure stages compared to control ($P < 0.02$), and decreased the AD duration during kindling acquisition compared to tripolar TFS before AK ($P < 0.01$). Animals that had tripolar TFS after AK application remained at the focal seizure stage, Stage II, for approximately 20 days after the cessation of tripolar TFS, beyond the 40 AK stimulations alone. We also performed a finite element model analysis to estimate what area what possibly activated in the brain. We estimate that the 2.5 mA TFS achieved electric fields greater than 0.3 mV/mm at depths less than approximately 12.6 mm deep into the brain. According to our measurements, 12.6 mm into the brain is sufficient depth to reach deep mesial structures, such as piriform cortex, amygdala, and hippocampus.

These results provide evidence that tripolar TFS induced antiepileptogenic and antiseizure effects when the tripolar TFS was administered after the AK. In addition, a long-lasting suppressive effect was observed; in this way, the animals remained at

the focal seizure stages of kindling for 20 days after tripolar TFS cessation, and approximately 80 AK stimulations were necessary to reach stage VI of kindling. It should be noted that the TFS was turned off after 40 days/treatments, but the AK stimulation was continued. In conclusion, tripolar TFS via TCRE applied during seizures over the epileptogenic area reduces seizure severity and retards epileptogenesis in cats.

26.4 Tissue Safety

26.4.1 Scalp

Besides being effective at abolishing or diminishing seizures, TFS must be safe for translation to clinical practice. We applied TFS to rat scalp and performed tissue histomorphological analysis to determine if the tissue was significantly damaged after TFS. We studied six different sets of stimulation parameters: 50 mA, 250 Hz, 200 μ s ($J^2t = 0.3$) ($A^2/cm^4 \cdot s^{-1}$), 50 mA, 200 Hz, 300 μ s ($J^2t = 0.7$) ($A^2/cm^4 \cdot s^{-1}$), 50 mA, 200 Hz, 500 μ s ($J^2t = 1.2$) ($A^2/cm^4 \cdot s^{-1}$), 100 mA, 250 Hz, 200 μ s ($J^2t = 1.2$) ($A^2/cm^4 \cdot s^{-1}$), 50 mA, 500 Hz, 300 μ s ($J^2t = 2.7$) ($A^2/cm^4 \cdot s^{-1}$), 50 mA, 500 Hz, 300 μ s, and 100 mA, 300 Hz, 500 μ s ($J^2t = 10.8$) ($A^2/cm^4 \cdot s^{-1}$) each for 60 s. Immediately after the end of TFS, we acquired a thermal image of the scalp. The maximum temperature measured from the rat experiments using ($J^2t = 0.7$) ($A^2/cm^4 \cdot s^{-1}$) low energy density stimulations was 38 °C. The maximum temperature measured from the experiments using ($J^2t = 2.7$) ($A^2/cm^4 \cdot s^{-1}$) high energy density stimulations was 47 °C. Stimulation at the energy density factor ($J^2t < 0.92$) ($A^2/cm^4 \cdot s^{-1}$) showed little or no damage. Some of the basal nuclei of the epidermis were more darkly stained than controls. The energy density factor ($J^2t 0.92\text{--}1.5$) ($A^2/cm^4 \cdot s^{-1}$) showed some moderate changes. The epidermal cells were less distinct at most of the stimulation sites. The nuclei were shrunken, darkly stained, and sometimes indistinct. The thickness of the epidermis appeared thinner than in the controls. We also observed damage to the hair follicles and sebaceous glands. Energy density factor stimulation ($J^2t > 1.5$) ($A^2/cm^4 \cdot s^{-1}$) showed more pronounced damage to the epidermis. The epidermis was compact and homogeneously stained, and no nuclei were present. The cells in the deeper epidermis were indistinct with darkly stained elongated nuclei. Collagen fibers in the dermis were clumped and appeared to be different in orientation compared with the control tissue. From this study, we concluded that as long as the specified energy density applied through the CRE was kept below ($J^2t < 0.92$) ($A^2/cm^4 \cdot s^{-1}$), the maximum temperature remained within the safe limits and also within the limits of the melting point of conductive paste and provided a safe current density distribution (Besio et al. 2010a, b).

26.4.2 *Cortex and Hippocampus*

We conducted further safety experiments on adult male Sprague–Dawley rats ($n = 60$; two groups of 30). We applied TFS (50 mA, 200 μ s, 300 Hz, 2 minutes, biphasic, charge-balanced pulses) via a TCRE. Group #1 received a single TFS application (9 controls, 21 TFS-treated). Group #2 received the same TFS on five consecutive days (9 controls, 21 TFS-treated). Cortical areas below the TFS site and hippocampal areas were assessed for neuronal damage. Following TFS application, rats were allowed to recover for 24 hours, one week, and one month, respectively. Then the rats were anaesthetized and transcardially perfused with 4% paraformaldehyde. The brains were removed, postfixed, sliced (30 μ m), stained (Nissl), and imaged (10 \times). No statistically significant difference was found in integrated optical density (IOD) values between the controls and TFS-treated rat brains for the three different latencies (t-test) (Mucio-Ramirez 2011). Morphological analysis did not show any pyknotic neurons or gliosis that might confirm any neuronal damage. Cell counts in the CA1, CA3, and dentate gyrus hippocampal areas also showed no significant difference. This suggests that TFS, under these conditions, is innocuous to the rat cortex and hippocampus.

26.4.3 *Safety of the Transcranial Focal Electrical Stimulation via Tripolar Concentric Ring Electrodes for Hippocampal CA3 Subregion Neurons in Rats*

The goal of this study was to assess the possible effect of TFS on hippocampal CA3 subregion neurons in rats. The rationale for the CA3 subregion analysis stems from it being one of the most important regions of the limbic system. In particular, studies suggest that the CA3 subregion of the dorsal hippocampus mediates the acquisition and encoding of spatial information within short-term memory with the duration of seconds and minutes (Kesner 2007). We used neuronal counting to quantify changes due to TFS since it is an established approach to assess the degree of neuronal loss as a measure of healthy neuronal density in the homogeneous CA3 subregion.

A single dose of TFS was applied (50 mA, 200 μ s, 300 Hz, biphasic, and charge-balanced pulses) for two minutes in one group and four more doses on consecutive days in the other TFS-treated group. The control group animals were all instrumented the same but received sham TFS, 0.0 mA, for two minutes. To test for different time delays and for tissue damage, 24 hours, 1 week, and 1 month after the last TFS treatment, the animals were perfused, and the brains were fixed. Coronal sectioning was performed at 30 μ m. Every fifth section containing the dorsal hippocampal CA3 subregion was collected. Slices were then mounted on gelatinized slides and Nissl stained. Slides were dehydrated, cleared, and mounted. Images were taken of the CA3 region, and counting was performed with ImageJ.

There was no significant difference in the cell counts in the CA3 region between the TFS-treated groups and the controls. At the TFS parameters tested, we did not see any neuronal damage in the CA3 region when applied acutely, and one does, or repeatedly for five consecutive days (Mucio-Ramirez and Makeyev 2017).

26.4.4 Transcranial Focal Electrical Stimulation Via Tripolar Concentric Ring Electrodes Does Not Modify the Short- and Long-Term Memory Formation in Rats Evaluated in the Novel Object Recognition Test

In the previous safety testing, we performed histological analysis to evaluate tissue from different locations in the path of the TFS. The tissue was taken at specific points in time and staining was performed. None of the prior analysis evaluated behavioral changes in the functioning brain. This study evaluated the effects of TFS via TCRES on the memory formation of healthy rats as a safety test of TFS. Short- and long-term memory formation was tested after the application of TFS using the novel object recognition (NOR) test (Rogel-Salazar 2013; Luby Matthew et al. 2014). The short- and long-term side effects of TFS are not completely understood. It is possible to study the safety of electrical stimulation in the brain through the analysis of its functional consequences on memory formation. We hypothesized that TFS via TCRES has no undesirable effects on memory formation and is safe per se. The aim of this study was to evaluate the effects of the TFS via TCRES on the memory process of healthy rats. To explore this issue, we addressed the following question: what are the functional consequences of applying noninvasive TFS via TCRES on the short- and long-term memory formation, as tested in the NOR test, in healthy rats?

Sprague–Dawley rats weighing between 250 and 300 g were the subjects of the investigation. Rats were habituated to handling for a week. The NOR test was performed in a blue acrylic opaque open-field chamber (60 × 60 × 60 cm) (Clever System Inc.) with faint black painted squares (15 × 15 cm). A video camera mounted directly above the box was used to record the testing session and to evaluate the behavior. Objects were placed in the open field, and the rats were allowed to become familiar with the objects. The rats were then taken out and either received TFS, 300 Hz, 200 us biphasic square pulses at 50 mA for two minutes or sham TFS, 0.0 mA. One of the objects in the chamber was replaced with a novel object. The rats were then put back into the open field. The rats were then removed from the open-field chamber, and a new novel object was entered prior to the rats return to the open-field chamber at 10 s, 1 min, 10 min, 90 min, 24 h, and 48 h. We tested three groups, naïve controls (no TFS), control who received sham TFS, and the TFS group ($n = 12, 12, 13$, respectively).

We used the recognition index (RI) to evaluate cognitive function. The RI was calculated by dividing the novel object exploration time by the total exploration

time (novel/(novel+familiar investigation)). Values of RI close to 0.5 indicate that animals spent equal time exploring both objects (familiar and the novel), while RI values greater than 0.5 denote a preference to explore the novel object over the familiar one. All three groups showed more preference for exploring the novel object than the familiar one at all the delay times. We found that the TFS via unique TCRES does not modify the short- and long-term memory formation in healthy rats as evaluated with the NOR test. These results suggest that short and long-term memory formation is not affected by the TFS.

26.4.5 Transcranial Focal Electrical Stimulation (TFS) via Tripolar Concentric Ring Electrodes (TCRES) Safety in Humans

After all the successful safety studies of TFS in rats, we finally received approval to perform a preliminary safety study on humans. Twenty-one individuals volunteered to participate in the three-session, double-blind control study (McCane 2018a, b). We chose a between-groups design with random intervention assignment since TFS's effects on people have never been tested. Five domains were tested for safety in three ~1-hour sessions conducted about 1 week apart: skin condition, participant sensation, cognition, and behavior using a working memory task (accuracy and reaction time), and electrophysiology via EEG frequency analysis (Fig. 26.5a). The participant and experimenter collecting the data were unaware of the experimental condition (TFS or Sham); a second experimenter predetermined the condition for each participant and delivered the TFS or Sham (double-blind control study).

Skin condition was measured at five individual locations on the arm and scalp for each progressive step in the electrode application process and subsequent stimulation (TFS or Sham). This detailed evaluation only occurred during Session 1. Each step was applied at a new arm or scalp location to isolate its sensations. The steps were (in order of application): (1) cleansing with NuPrep gel alone (Weaver and Company, Aurora, CO, USA), (2) application of Ten20 EEG paste alone (Weaver and Company, Aurora, CO, USA), (3) cleansing plus Ten20 paste (in that order), (4) cleansing plus a TCRES loaded with Ten20 paste (3 mm depth), and (5) cleansing, application of TCRES with Ten20 paste, then TFS or Sham for 2 minutes. Immediately following each step, the participant used a visual analog scale to rate pain (VAS, 0–10, no pain – worst pain ever) and was asked to report any other sensations they experienced. The experimenter rated skin erythema based on the Draize scale (0–4, no change – beet red). We used a working memory task (hereafter, the 'task') to measure human behavior and cognition changes. Each task block consisted of one set of a 2-back and 3-back task (~4 mins/set) with approximately one minute of rest between sets. There were two sets of each task to reduce memorization. The participants' start version and type (2- or 3-back) were counterbalanced. One block of baseline task was performed before scalp TFS or Sham at Session 1. Four blocks were collected

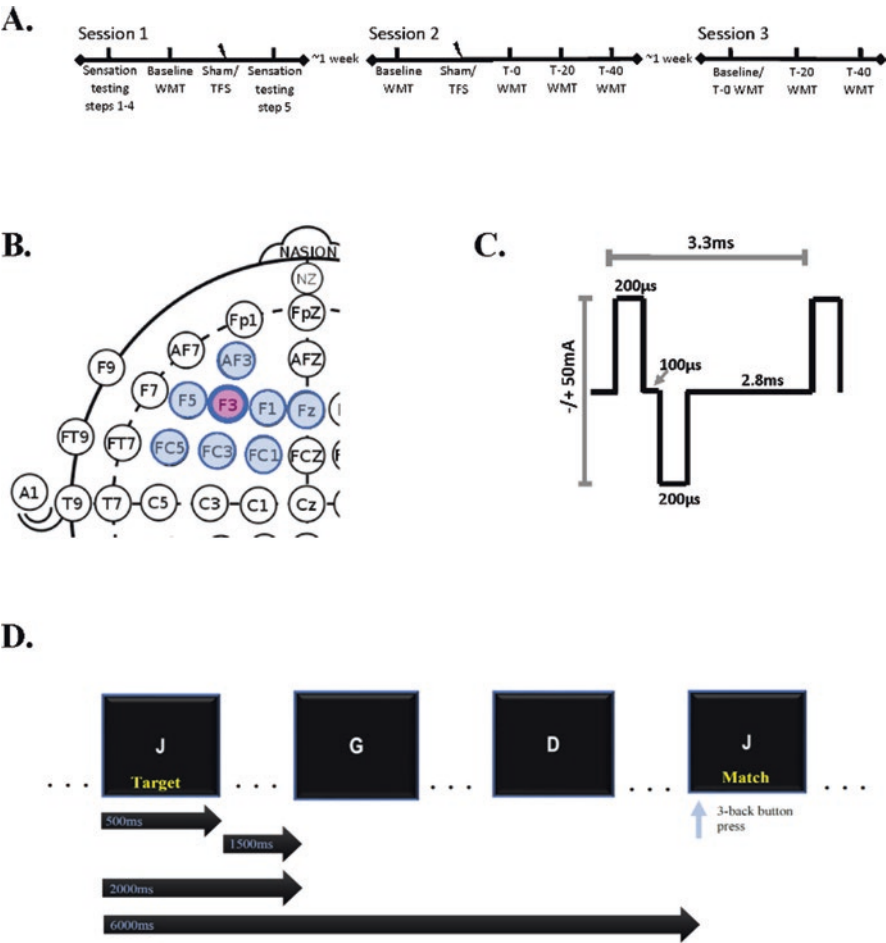


Fig. 26.5 (a) Shows what happened during the three sessions that healthy humans participated in the study. (b) Shows the locations that were recorded from, around the F3 location that was used for recording and stimulation. (c) The TFS pattern. (d) A depiction of the n-back test as seen by the participant on the screen in front of them

at Session 2: one baseline block prior to TFS or Sham and three postintervention blocks at three timepoints: 0-, 20- and 40-minutes. The selection of these timepoints were based on other tES dosage studies (Hoy 2013; Choe 2016). Session 3 was conducted to evaluate the stimulation’s possible long-term effects, and no TFS or Sham was administered. Three blocks were collected (0-, 20- and 40-minutes) at Session 3, and the 0-minute block was also used as the session baseline.

Each task set displayed one of ten semantic items (A-J of the English alphabet) 150 times (25% targets) for 500 ms followed by a blank screen for 1500 ms before the next item was displayed (Fig. 26.5d). Four or six seconds passed between target

and matching items (2- and 3-back trials, respectively). The items were presented uninterrupted; every item could have a potential match, and the participant was presumably engaged until the 148th and/or 149th items, depending on the task.

TFS's potential effects on brain electrophysiology were measured using electroencephalographic (EEG) signals recorded with TCREs at eight locations over the left dorsolateral prefrontal cortex (AF3, F5, F3, F1, FZ, FC5, FC3, FC1, Fig. 26.5b). The construction of TCRE electrodes allows them to collect standard EEG and tEEG simultaneously. The eight tEEG signals were first passed through a t-Interface 20 2.0 preamplifier (CREmedical, high pass 0.3 Hz), then to a 16-channel g.USBamp (g.tec medical). The eight EEG signals emulated by the outer ring of the TCRE were passed through the t-Interface 20 and then sent to the g.USBamp. The signals were recorded at 512 Hz using BCI 2000 (Schalk 2004) for signal acquisition, task presentation and subject response coding. The intervention Session 2 signals (EEG and tEEG) at the site of stimulation (F3) were evaluated in MATLAB (Mathworks 2017b).

The subjective sensory effects on the forearm and scalp were rated by the participants using a visual analog scale (0–10, no pain–worst pain ever). The arm and scalp scores for each participant were combined ($n = 42$) since most scores were zero and there were no scores >1 . The experimenter rated skin erythema for each step of electrode application including TFS or Sham (Draize 0–4, no change–beet red) at Session 1, inspected the application sites before and after intervention at Session 2, and again before Session 3. Erythema results are based on combined arm and scalp scores ($n = 42$). Most participants had no erythema response to any application step, including TFS or Sham. Combined, 96% received '0's ratings for erythema; only nine '1's were reported of 210 total scores. In summary, the TFS application process tested in this study did not cause immediate or delayed dermatological damage in any participant and produced no sensation in the majority of healthy TFS participants. Working memory task accuracy and reaction time were used to assess the cognitive and behavioral effects of TFS compared to sham. The results of the 2- and 3-back working memory tasks within each of the eight blocks were averaged (\pm SD) for each subject and then for each group (\sim 300 trials/block/participant, 25% targets). There were no significant differences in accuracy or reaction time (Wilcoxon rank sum) between TFS and Sham participants for any of the eight task blocks. The mean EEG and tEEG power ratios at the stimulation location (F3) during Session 2 for each group were used to test potential acute TFS cortical effects. Three time-points (time-0, time-20, and time-40) postintervention were compared (t-test) for each signal type (tEEG and EEG) using 3 Hz bins (0–36 Hz). There were no significant differences between the TFS and Sham groups for either signal at any bin. From these results, the preliminary safety study showed there was no significant difference between sham and TFS groups inferring that TFS is safe in humans.

26.5 Concluding Remarks

In summary, we have shown the efficacy and safety of TFS in rats. In four different acute rat seizure models, we have shown that TFS is effective. We first showed that TFS significantly reduced penicillin-induced myoclonic jerks in rats (Besio 2009; Besio et al. 2010a, b). We then showed that TFS significantly reduced the risk of death due to pilocarpine-induced status epilepticus and had a long-lasting effect (Besio et al. 2007). We also showed that TFS reduced the brain electrographic synchronization caused by PTZ (Besio 2011a, b). Further, we found that TFS significantly reduced the PTZ-induced brain electrographic power and duration of myoclonic jerks (Besio et al. 2010a, b; Makeyev 2011). We have also shown that an automated noninvasive seizure control system was possible utilizing TFS (Makeyev 2012). Though all of the models we have utilized to show the effectiveness of TFS have been acute seizure animal models, we believe that TFS will also be effective in the epileptic brain. We recently showed that we could revert the DRS with TFS (Perez-Perez 2021). Another interesting finding was that we could delay, and prevent, brains from becoming kindled (Valdés-Cruz 2019). We also found, from the amygdala kindling experiments, that there was a long-lasting effect from the TFS. Even 20 days after the TFS was stopped, and the kindling was continued each day, the cats did not advance beyond level two, the same level they were during the days of TFS (Valdés-Cruz 2019). Beyond seizure control, we have also shown the safety of single and multiple doses of TFS. We initially showed that TFS was safe to rat scalp, if the energy density factor was kept below $0.92 \text{ A}^2/\text{cm}^4 \cdot \text{s}^{-1}$ (Besio et al. 2010a, b). Since brain tissue may be more sensitive than the scalp, we then analyzed TFS-treated brain tissue. Our preliminary results show that TFS, in a single dose or in multiple doses, does not cause any significant difference in the rat cortex or hippocampus (Mucio-Ramirez 2011). We then showed that TFS did not alter short- and long-term memory in rats (Rogel-Salazar 2013). Lastly, we showed preliminary safety of TFS on human short- and long-term memory as well (McCane 2018a, b). These findings all suggest that TFS is efficacious for controlling acute seizures in rats and does not cause significant safety concerns. Beyond controlling acute seizures, the results from our animal experiments with DRS have shown that TFS is able to revert DRS and may hold promise for humans (Perez-Perez 2021). Future studies must be designed to evaluate if this strategy is able to reduce seizure activity in pharmacoresistant epilepsy human patients.

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